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Characterization and exploitation of a marine microbial culture collection

– a special focus on carotenoid producing heterotrophic bacteria

Thesis for the degree of Philosophiae Doctor

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Norwegian University of Science and Technology
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Preface

The present PhD study has been conducted at The Department of Biotechnology at Norwegian University of Science and Technology (NTNU) and SINTEF, Department of Biotechnology and supported by a grant from the Norwegian Research Council and NTNU. This project has been a collaboration between SINTEF, NTNU and the industrial partner Promar AS.

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Adventure is just bad planning ~ Fridjof Nansen

Trondheim, April 2013
Marit H. Stafsnes

Abstract

Marine microorganisms are regarded promising sources of bioactive molecules. For this reason, a marine heterotrophic bacterial collection from the sea surface microlayer had been established intending to identify molecules with potential commercial interest. To exploit this bacterial collection, rational screening procedures have to be developed. A high-throughput LC-MS protocol was established for screening of pigmented bacteria, rapidly characterizing the UV/Vis properties of the pigments. Between one and ten distinct pigments were identified in each bacterial isolate, with the majority of isolates producing three to five pigments. Carotenoids were the focus of this study, in particular carotenoids absorbing light in the upper UVA/visible light area. This type of carotenoids can be valuable as sunscreen agents, complementing the protection where conventional sunscreens offer low or no protection. In this study, several potential producer strains were identified.

Among several bright yellow colored bacterial strains from the culture collection, one formed more intensively colored colonies than the others did. The isolate, designated Otnes7 was determined to be a *Micrococcus luteus* strain, and LC-MS analysis of extracted pigments revealed the C_{50} γ−cyclic sarcinaxanthin as the major accumulated carotenoid. *In silico* screening of the genomic sequence data of *M. luteus* strain NCTC2665 resulted in identification of a putative carotenoid biosynthesis gene cluster. The genes in this cluster were putatively encoding for GGPP synthase (CrtE), phytoene synthase (CrtB), phytoene desaturase (CrtI), lycopene elongase (CrtE2), C_{50} γ-cyclase subunit (CrtYg), C_{50} γ-cyclase subunit (CrtYh), and glycosyl transferase (CrtX). The complete *crt* gene cluster was cloned from strain Otnes7 and NCTC2665 in *Escherichia coli* hosts, and the sarcinaxanthin biosynthetic pathway was experimentally elucidated. Partial expression of the gene clusters were performed to reveal the single steps in the sarcinaxanthin biosynthesis. Carotenoid analysis from the resulting strains by LC-MS and NMR revealed that sarcinaxanthin is synthesized from the precursor farnesyl pyrophosphate via the intermediates lycopene, nonaflavuxanthin and flavuxanthin. Eventually 10-20 % of the sarcinaxanthin is glycosylated by CrtX.

Heterologous expression of the genes in *E. coli* by using the adjustable Pm promoter resulted in up to 10-fold higher sarcinaxanthin production levels compared to the levels obtained by *M. luteus* strains under comparable laboratory conditions.

Flavuxanthin is also an intermediate in the biosynthesis of the structurally related ε-cyclic decaprenoxanthin. In an attempt to understand the specific difference between the biosynthetic pathway of sarcinaxanthin and decaprenoxanthin, a hybrid gene cluster combining the lycopene elongase gene (*crtE2*) from *M.luteus* Otnes7 with the two genes encoding for C_{50} carotenoid cyclase subunits (*crtYe* and *crtYf*) in the decaprenoxanthin producing organism *Corynebacterium glutamicum* was constructed. Surprisingly, three different C_{50} carotenoids were identified in this construct; decaprenoxanthin, sarcinaxanthin and a new C_{50} carotenoid denominated sarprenoxanthin. These data contribute to new insight into the diverse and multiple functions of bacterial C_{50} carotenoid cyclases and these cyclases should therefore provide attractive targets for pathway diversification and directed genetic engineering to generate novel carotenoids.

In an effort to develop rational screening tools for identification of novel pigment producers in the marine bacterial collection, MALDI-TOF-MS was explored as a dereplication tool. MALDI-TOF-MS analysis and subsequent creation of similarity based dendrogram from nearly 400 strains from the bacterial collection showed that the bacterial collection is a diverse collection of marine heterotrophic bacteria. The pigmented bacteria showed greater diversity than the non-pigmented bacteria. Pigment extracts from a selection of the pigmented strains was analyzed with LC-DAD-MS. A dendrogram based on their pigment profiles was created. The pigment profile dendrogram was then linked to the corresponding MALDI-TOF MS dendrogram. These results show that pigment profiles can be used as taxonomic markers when the isolates produce at least three different pigments. In addition, MALDI-TOF MS can be used as dereplication tool to avoid redundant analysis without compromising the diversity of the collection when screening for novel pigmentation.

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Paper I

Stafsnes, M, K Josefsen, G Kildahl-Andersen, S Valla, T Ellingsen, and P Bruheim. 2010. Isolation and characterization of marine pigmented bacteria from Norwegian coastal waters and screening for carotenoids with UVA-blue light absorbing properties. The Journal of Microbiology 48(1):16-23.

Paper II

Netzer, R*, MH Stafsnes*, T Andreassen, A Goksøyr, P Bruheim, and T Brautaset. 2010. Biosynthetic Pathway for γ-Cyclic Sarcinaxanthin in *Micrococcus luteus*: Heterologous Expression and Evidence for Diverse and Multiple Catalytic Functions of C50 Carotenoid Cyclases. Journal of Bacteriology 192(21):5688-5699.

*) Contributed equally to this work.

Paper III

Stafsnes, MH, M Dybwad, A Brunsvik, and P Bruheim. 2013. Large scale MALDI-TOF MS based taxa identification to identify novel pigment producers in a marine bacterial culture collection. Antonie van Leeuwenhoek 103(3):603-15

Paper IV

Stafsnes, MH and P Bruheim, eds. 2012. Pigmented Marine Heterotropic Bacteria: Occurrence, diversity and characterization of pigmentation. In Marine Biomaterials: Characterization, Isolation and Application. ch 9. ed. K Se-Kwon. Taylor & Francis Group, LLC. Accepted for publication.

Symbols and abbreviations

A1%/1cm	absorbance at λmax of a 1% solution in a 1 cm cuvette
APCI	atmospheric pressure chemical ionization
BAC	bacterial artificial chromosome
BLAST	basic local alignment search tool
CDW	cell dry weight
CFU	colony forming units
CrtB	phytoene synthase
CrtE	GGPP synthase
CrtI	phytoene desaturase
DAD	(photo)diode array detector
DMAP	4-dimethylamino pyridine
DMAPP	dimethylallyl diphosphate
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNP	Dictionary of Natural Products
DOXP	1-deoxy-D-xylose-5-phosphate
Dxr	1-deoxy-d-xylulose 5-phosphate reductoisomerase
Dxs	1-deoxy-D-xylulose 5-phosphate synthase
FDA	Food and Drug Administration
FPP	farnesyl diphosphate
G3P	D-glyceraldehyde-3-phosphate
GGPP	geranylgeranyl pyrophosphate
HMBPP	(E)-4-hydroxy-3-methyl-butenyl-pyrophosphate
HPLC	high performance liquid chromatography
HTS	high-throughput screening
IPP	isopentenyl diphosphate
IR	infrared light
LC-MS	liquid chromatography/mass spectrometry

MALDI matrix assisted laser desorption ionization

MEP 2-*C*-methyl-o-erythritol-4-phosphate

MVA mevalonic acid

NMR nuclear magnetic resonance

NGS next generation sequencing

-OH hydroxyl

PCR polymerase chain reaction

PHB pigmented heterotrophic bacteria

rRNA ribosomal ribonucleic acid

RP reverse phase

SIM single ion monitoring

SML sea surface micro layer

SPF sun protection factor

SQ single quadrupole

TLC thin layer chromatography

TOF time of flight

UV ultraviolet light

UW underlying water

Vis visible light

λmax maximum absorption wavelength

1 Introduction

1.1 Bioprospecting and Natural products

Humanity has a long history in the search for useful and potentially valuable natural products. This search was termed bioprospecting in the early 1990s [42] and is defined as the search for useful organic compounds in nature with the intent of developing products of commercial or social value. Within bioprospecting, the search for antibiotics and drugs for anti-cancer treatment have received most attention. However, there are many natural products with other possible uses in medicine, neutraceuticals or cosmetics. Our research has been focused on valuable pigments and in particular carotenoids as the most widespread group among natural pigments.

In general, natural products are chemical compounds or substances produced by any living organism. However, in a bioprospecting context, they are in most cases referred to secondary metabolites, defined as organic compounds that are not necessary for the survival, growth, development, or reproduction of the organism that produces them. Concepts of secondary metabolism include products of overflow metabolism as a result of nutrient limitation, or switch metabolism after the active growth phase, defence mechanisms, or regulator molecules [37]. These secondary metabolites may be conserved for a relatively long period and with time may come to confer a selective advantage to the producing organism [288]. This is supported by the fact that secondary metabolites are often unique to a particular species or group of organisms [21]. Often secondary metabolites exhibit some kinds of biological activities, i.e. they are molecules that have an effect upon a living organism or on living tissue and these are referred to as bioactive secondary metabolites.

Natural products played a prominent role in ancient traditional medicine systems such as Chinese, Ayurveda and Egyptian and are still commonly used for treatment of various diseases; fifty percent of all approved drugs in 2010 were of natural origin [181]. Also their uses as colorants, spices, fragrances, aphrodisiacs, cosmetics, and toxins have been fundamental to human culture and development [182]. Several well known plant species, e.g. licorice (*Glycurrhiza glabra*), myth (*Commiphora species*) and opium poppy (*Papaver somniferum*) are mentioned as medicinal herbs in the first

known written record on clay tablets from Mesopotamia in 2600 BC [47]. Egyptian medicine dates from about 2900 B.C., and the best known Egyptian pharmaceutical record is the Ebers Papyrus dating from 1500 B.C., describing 700 plant-based drugs [47].

The natural product history is not only about pharmaceutical products are part of; the production of colors was of great cultural and economic importance. One of the most important colors was Tyrian Purple, known already in the 4th century BC. The dye is a bromine compound and originates from hypobranchial glandular secretions of *Murex trunculus*, a predatory sea snail found in the eastern Mediterranean. Huge quantities of snails were required and spoil heaps of the shells can still be seen on the sites of ancient dye works around the Mediterranean. In 1908, Friedlander collected just 1.4 grams of pure dye from 12,000 mollusks [157]. The natural dye known as indigo was obtained from the leaves of the *Indigofera tinctoria* plant originating in India (4th century BC) and contains an indigoid compound [94]. The production of Indian Yellow, however, was closer to modern biotechnological production; cows were fed exclusively on mango leaves and the resultant urine was dried to obtain the pigment. This led to serious malnutrition for the cows and the practice was outlawed in 1908 [248]. The pigment was identified as a xanthonoid, a natural phenolic compound [63]. Another important natural pigment is the brown pigment sepia. The main constituent of sepia is melanin and it was obtained from various cuttlefish from the 16th century [150]. After the accidental synthesis of aniline purple by William Henry Perkin in 1856, modern pigment chemistry started and left no space for the continuous search for natural pigments.

At the same time developments in chemistry allowed scientists to examine plants in order to understand why they were medically useful [23]. Morphine was the first identified pure natural product with documented bioactivity [229]. Other examples of bioactive natural products are quinine [158] and coca [11]. With the discovery of penicillin and it's antibacterial activity by Fleming in 1929 [72] and its isolation by Chain and Florey in 1940 [40], the focus shifted to natural product screening from microorganisms [233]. Microbes from soil samples were cultured and identified in extensive screening programs, yielding many antibiotics that are still in use today. By 2002, over 22,000 microbial bioactive compounds had been discovered [53]. Between

1970 and 2000, however, no novel classes of antibiotics were licensed [282] and since 2000, only two new classes of antibiotics derived from natural products have been approved [36].

While the discovery of new antibiotics slowed down, the attention turned to searching for potential anti-cancer drugs as it was realized that compounds possessing antibiotic activity also possess other types of bioactivities. Microbial secondary metabolites were proven to be a valuable source of toxins, effectors of ecological competition and symbiosis, pheromones, enzyme inhibitors, immunomodulating agents, receptor antagonists and agonists, pesticides, antitumor agents and growth promoters of animals and plants, and pigments [54]. The outcome of these new screening programs were limited, and most pharmaceutical companies turned their focus to "rational drug design" using combinatorial chemical synthesis [19]. Rational drug design is not based on trial and error as screening for bioactive molecules, but involves the design of small molecules that are complementary in shape and charge to the molecular target with which they interact and therefore will bind and inhibit the biological function of the target. The approach gained a lot of attention, and a major victim was natural-product screening. This did not turn out remarkable successful either and perhaps one has to acknowledge the fact that the nature provides novel molecular skeletons with a complexity that seems impossible to achieve through combinatorial chemical synthesis [23].

Development in technologies for separation science, spectroscopic techniques, microplate-based bioassays and high-throughput screening (HTS) have made natural products research gain momentum in recent years [223]. The identified natural products from these screening programs usually function as starting material for subsequent chemical or microbiological modifications or function as lead compounds for chemical synthesis of new analogs or as templates for drug design in order to improve their properties [21].

1.2 Marine microorganisms as an emerging source of novel bioactive compounds

New terrestrial natural bioactive compounds are no longer discovered so frequently although it is estimated that up to now only 5-10 percent of terrestrial plants have been explored for drug discovery [223]. Instead, water-based ecological systems are regarded as more promising sources. In particular, marine microbes are a promising source for bioactive molecules. Complex ecological pressures and extreme conditions have driven microbial evolution in the sea for over 3.5 billion years resulting in extraordinarily varied microbial adaptation. The great variation in abiotic factors (e.g. pressure, salt, temperature, light, nutrition) leads to the creation of unique habitats in the marine environment. Marine microorganisms may consequently have evolved a greater range of novel physiological and chemical capabilities than terrestrial [52, 57, 61, 205]. For instance some Gram-negative *Pseudomonas* and Gram-positive *Bacillus* species of marine origin have been reported to produce metabolites with extraordinary structures, different from those isolated from corresponding terrestrial species [102, 106, 276].

The first bioactive components from the marine environment were isolated in the early 1970s [71]. Since then a wide variety of bioactive secondary metabolites and enzymes have been isolated from marine microorganisms [149, 184]. Many pharmaceutical companies are now concentrating their natural products research on marine microorganisms [24].

1.2.1 The sea surface microlayer and the bacterioneuston

The sea surface microlayer (SML) is defined as the top millimeter of the ocean surface. It is the boundary layer where exchange between the atmosphere and the hydrosphere occurs. The SML can be divided in distinct layers (Figure 1.1). From the top it has a monomolecular lipid film, then a polysaccharide-protein layer, a layer of suspended abiotic particles, and bacterioneuston and deeper layers of phytoneuston (mainly algae) and zooneuston [296].

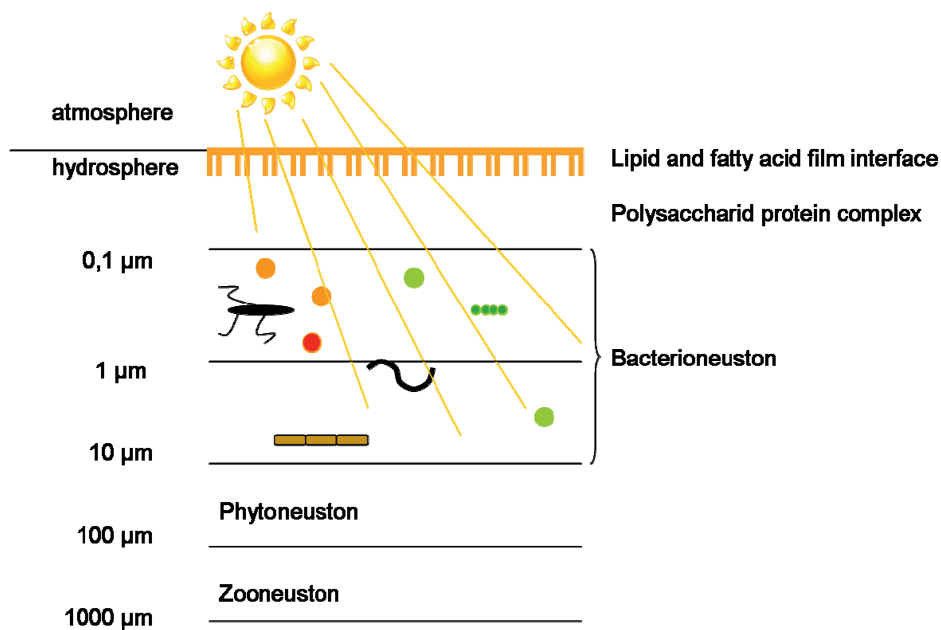

Figure 1.1 Schematic representation of the surface microlayer stratification adopted from [92, 296].

The physical, chemical, and biological properties of the SML are distinctly different from the underlying water (UW) [148]. Chemical contaminants accumulate in the SML due to its unique physicochemical properties, thereby creating a substantial unpredictability of chemical characteristics, compared to the UW. Therefore, the SML has often been considered an extreme environment for microorganisms that may contain unusual species and taxa that have developed life strategies to survive in this habitat [155]. On the other hand, the SML is generally enriched in organic material (up to 1000 times higher concentration than UW), and this might fuel bacterial growth and the development of microbial food webs. Therefore, higher abundances of microorganisms have frequently been reported for the SML than the UW, often 10-100 times higher [38, 92, 148, 239]. However, lower microbial concentrations in the SML have also been observed [20]. The SML and the organisms living there are at times exposed to intense light- and UV-radiation from the sun, and microorganisms sustaining these levels have a competitive advantage. One way of protecting themselves from radiation is by

producing pigments such as carotenoids that can protect the cells by stabilizing the cell membrane.

1.2.2 Microbial diversity and culturability

The industrial microbiology field has utilized only a small portion of nature's microbial arsenal for the discovery of useful molecules. One main reason is the inability of microbiologists to culture the vast majority of microbes in nature. The uncultured or yet-to-be cultured microorganisms are estimated to account for almost 99% of all soil microbiota and nearly 99.9% of marine microorganisms. This phenomenon, called "the great plate count anomaly" [249], has been known for more than 30 years [96].

Metagenomics proves the lack of culturability

Metagenomics is defined as the direct genetic analysis of genomes contained with an environmental sample [264]. Metagenome libraries are created by cloning genomic DNA from environmental samples [208]. These libraries can then be screened for functional expression and the genes are sequenced with shotgun DNA sequencing, a DNA sequencing technique which requires the target DNA to be broken into random fragments [274]. This method is however very labor-intensive and the cloned genes might be toxic for the cloning host [244]. Next-generation sequencing (NGS) technologies does not require a metagenome library and combined with decreasing costs and improved bioinformatics tools for gene annotation and assembly NGS is therefore gradually replacing the shotgun DNA sequencing technology [264].

For taxonomic studies, usually the 16S rRNA gene sequences are used. As expected, many more microbial species than those culturable under laboratory conditions, have been described [12, 110, 267] proving the lack of culturability of microbial species [90]. Of the strains described by metagenomics, many were not even closely related to isolated strains and several candidate phyla have been proposed [110, 226]. Currently there are 30 distinct bacterial phyla listed with cultivated members and in addition 70 candidate phyla based solely on 16S rRNA gene sequences [3, 67, 99]. Metagenomics has also given new insight into understanding the abundance of different bacteria [204]. The most abundant of cultivated bacteria belong to Gammaproteobacteria, whereas Alphaproteobacteria are most abundant in natural

environment as revealed by 16S rRNA. One very abundant group of bacteria, representing on average one third of the microbial cells in the ocean surface, the SAR11 clade, was only discovered in 1990 [173] and cultured in 2002 [203].

Metagenome libraries are used to screen for particular DNA sequences or for functional expression of the cloned genes. Particular phenotypes, e.g. pigment production are found by visual inspection. Identified functions from metagenomes (e.g. proposed metabolic life strategies) can again be used to develop cultivating strategies for uncultured species, [8, 89].

Strategies for increasing the culturability of microorganisms

Although metagenomics can reveal functions of single enzymes or entire biosynthetic pathways, the characterization of physiological properties and understanding of metabolic and energetic capabilities can only be achieved through the isolation of individual bacterial species in pure culture [273]. Hence, for years microbiologists have been working on developing new ways to culture environmental microorganisms in attempts to overcome the "great plate count anomaly" [108, 204, 251, 290]. In general, aiming to mimic the growth conditions from the natural environment is likely to result in higher rates of cultivated bacteria. Several strategies for natural environment mimicking have been developed and the most important are discussed.

The use of very low nutrient concentrations, often in combination with long periods of incubation will favor growth of slow-growing bacteria. Particularly microbes that originate from oligotrophic habitats where a non-growing or dormancy state may be the norm might easily be discarded before visible growth is obtained and incubation periods of several months might be required [4, 279]. Higher concentrations of substrate may even be toxic. Connon and Giovanni were able to cultivate up to 14% of the cells from coastal seawater by using this approach [44], and members of the abundant SAR11 clade bacteria was for the first time isolated by using incubation times up to 24 weeks combined with low-nutrient media [44, 203]. The principal behind this approach is to allow growth of only the desired microbes. Unwanted microbes can also be inhibited with salts, dyes, or other chemicals.

Other more specific approaches are encapsulation of cells directly from environmental samples in gel microdroplets and thereafter detection of microcolonies

by flow cytometry [290, 291]. Combined with environmental concentration of nutrients, single encapsulated cells grow and form microcolonies within the microcapsules and the fast growing species will burst the microcapsules. This method has been suggested to be suitable for massively parallel cultivation of microorganisms for natural-product screening and drug discovery [110].

The traditional microbiological approach of selective culture does not allow for interactions between microbes that occur between organisms in the natural environment: the fastest growing species may overwhelm those that divide only very slowly, thus leading to an imbalance of cell-to-cell communications. By specifically targeting the isolation of consortia, rather than single species, it may be possible to bring many more environmentally relevant bacteria into laboratory culture. Incubation in diffusion chambers with simulated natural environment aims to include these factors [108]. The diffusion chambers separate the bacterial assemblage of interest from a source of nutrient utilizing a semi-permeable membrane. In this way, isolates that does not grow on artificial media alone but forms colonies in the presence of other microorganisms, can be cultivated. Kaeberlein and co-workers successfully cultivated 2–40 % of the inoculated cells by using this technique [108]. Growth factors produced by closely related organisms have been shown to greatly increase cultivation success. As much as 40 % of the total community from a marine sediment biofilm has been cultivated by using helper strains that produce growth factors identified as siderophores [50].

Even when the whole spectrum of nutrients and signalling factors are present for successful cultivation, presence of inhibitory compounds like bacteriocins in the isolated community might result in the inactivation of the cells by other microbes in the immediate vicinity [259]. Virus infection may prevent growth in culture; this may be either infection with phage or the change to the lytic cycle of temperate phages when nutrients are supplied to starved bacterial cells [68].

The advances made in culturing techniques are far from offering the possibility to culture all the microorganisms present in a an environmental sample, but increasing the culturability up to 40% is a massive improvement, that again improves the potential for natural products discovery.

1.3 Screening and Dereplication of large collections of bacteria

High throughput drug screening of bacterial collections started in the late 1980s. Robotic methods of sample handling and detectors capable of reading 96-well microtiter plates were developed, and the emphasis of screening shifted from empirical measures of cell growth or function to molecular targets [23]. With the increasing knowledge of genes and receptor biology, bioassays were developed and used in the screening process. The scope of the primary screen is to rapidly identify samples with bioactivity of the desired type from a large number of samples. "Secondary testing" procedures involve more detailed testing of lead compounds and these assays usually have low capacity, are slow and costly [16].

A major problem within natural product drug discovery programs is the frequent rediscovery of already known compounds, as compound isolation and structure elucidation is very time and resource demanding. Methods intended to rapidly identify known compounds in natural product extracts, is referred to as dereplication [46, 80]. This is an important step in an efficient drug discovery program. Introduction of modern spectroscopic methods and tandem analytical techniques, such as HPLC-DAD (high performance liquid chromatography-(photo)diode-array-detector, LC-MS (mass spectrometry), LC-MS-MS, LC-NMR (nuclear magnetic resonance), and LC-NMR-MS [22, 76, 183] (to be discussed later) have revolutionized compound identification and tremendously accelerated the pace at which isolated compounds can be identified. The identity of an active compound can be determined at an early stage in the discovery process by consulting databases for secondary metabolites. This prevents wasted effort on samples with no potential for development and allows resources to be focused on the most promising leads. Current databases for identification of natural products are presented in Table 1.1.

Table 1.1. Databases for natural product (NP) identification.

Database	Type of NP information	# of components	Reference
SuperNatural	3D structures	46 000	[62]
NMRShiftDB	NMR database	41 300	http://nmrshiftdb.nmr.uni-koeln.de/
COMET	Metabolite recognition	5 000	http://www.microbialscreening.com/
NAPROC-13	13C spectral information	6 000	[152]
DNP	Structure database	226 000	www.chemnetbase.com
AntiBase	1H NMR-structural features	50 000	[126]

1.3.1 Bacterial taxonomy in biodiscovery

Microalgae have been proven to contain division- or class-specific carotenoids and the production of carotenoids can therefore be used as a taxonomic marker [256]. However, the linkage between taxonomic and chemical diversity are not coherent for all microorganisms. The ability of horizontal gene transfer in *Streptomyces* was argued to be a proof that secondary metabolite production is strain specific [64]. This view excludes bacterial taxonomy as a useful tool for dereplicating bacterial strains in a biodiscovery setting. However there are several contraindications for this view; screening of a taxonomically dereplicated collection of actinomycetes led to a high number of discovered compounds compared to the strain throughput [84], a set of secondary metabolites as phenotypic markers have been found to be of general validity in fungi [134], and by marine invertebrate-associated bacteria [98]. Hence, species specific chemoprofiles can exist and bacterial taxonomy has been used for guided discovery of secondary metabolites in filamentous fungi [75].

Matrix assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) has become an important tool in bacterial identification and species differentiation. MALDI-TOF can be used for fast differentiation of isolated culturable bacteria based on their mass fingerprints (phyloproteomics). In the last few years MALDI-TOF MS has been increasingly studied and applied for the identification and typing of microorganisms [136, 216] and has also been proven valuable as a dereplication tool [4, 7].

1.4 Pigmentation in marine heterotrophic bacteria

Pigments are part of the secondary metabolite arsenal in microbes and are often associated with bioactivity. The pigment itself can be bioactive (Table 1.2) or it can be co-expressed with bioactive secondary metabolites. Therefore, several bioprospecting projects looking for bioactive molecules have been targeted to pigmented marine bacteria [73, 202, 243, 245]. Reported values of pigmented heterotrophic marine bacteria (PHB) varies significantly; from 10 to 70 % of total colony forming units (CFU), comprising primarily the colors red to yellow [39, 61, 95, 294, 295]. The huge variance in PHB can be due to different habitat, but most likely, a great deal of the discrepancy can be attributed to different isolation techniques (sampling techniques, media, and cultivation conditions). PHB are particularly frequent in the SML, and a plausible explanation for the pigment production is that pigmentation function as protectants for UV radiation [61, 95, 268]. Figure 1.2 shows isolated bacteria from the SML with typical pigmentation.

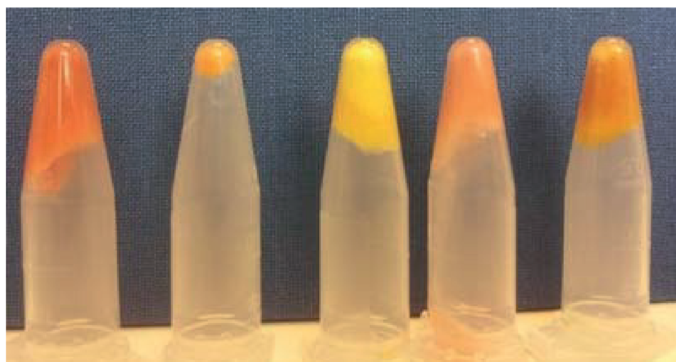

Figure 1.2. Example of pigmentation occurring in marine heterotrophic bacteria. Pellets from bacterial cultures isolated from the surface microlayer.

The most important pigment group found in marine heterotrophic bacteria are the carotenoids and is described in the next paragraph. Other important pigment groups found in marine bacteria are listed in Table 1.2 together with their biological function and production organism. A thorough description of pigments produced by marine heterotrophic bacteria can be found in paper IV; “Pigmented Marine Heterotropic Bacteria: Occurrence, diversity and characterization of pigmentation”.

Table 1.2. Pigments (groups) isolated from marine bacteria.

Pigment	Activity	Bacterial strains*	References
Astaxanthin (carotenoid)	Protect from UV radiation; Membrane stabilizer; Antioxidant	*Paracoccus haeundaensis* *Altererythrobacter ishigakiensis* *Agrobacterium arantiacum*	[46-48]
Prodiginines	Antibacterial; Anticancer; Algicidal	*Hahella chejuensis* *Serratia marcescens* *Pseudoalteromonas rubra* *Streptomyces coelicolor* *Zooshikella rubidus*	[112, 139, 283]
Violacein	Antibiotic; Antiprotozoan; Anticancer	*Pseudoalteromonas luteoviolacea* *Collimonas sp.* *Chromobacterium violaceum* *Janthinobacterium lividum*	[87, 146, 192, 284]
Tambjamines	Antibiotic, Anticancer	*Pseudoalteromonas tunicata*	[73]
Melanines	Protect from UV radiation	*Vibrio cholerae* *Shewanella colwelliana* *Alteromonas nigrifaciens* *Pseudomonas aeruginosa* *Marinomonas mediterranea*	[200, 243]
Phenazine derivatives	Cytotoxic	*Pseudomonas aeruginosa* *Brevibacterium sp.* *Bacillus sp.* *Pelagibacter sp.*	[13, 41, 230]

*) Not a complete list, see paper IV for a thorough description of pigments in marine heterotrophic bacteria

1.5 Carotenoids

Carotenoids are a subfamily of isoprenoids (also denoted terpenes), which are among the most widespread of all natural products. Isoprenoids are a varied class of hydrocarbons that function as building-blocks for important organic compounds such as resins, steroids, vitamins and essential oils. Carotenoids are responsible for most of natural red, orange and yellow coloration of plants and microorganisms as well as the colors of some birds, insects, fish, and crustaceans [143]. Over 750 structurally distinct carotenoids are known [34, 103], and new structures continue to be reported [191, 250, 258]. Carotenoids are divided in two main chemical groups, carotenes and xanthophylls. The latter are carotenoids containing hydroxyl (-OH) groups [34]. Carotenoids can also contain sugar units and are termed glycosylated carotenoids. The first carotenoid glycoside was isolated from saffron and described in 1818 [15]. So far few glycosylated carotenoids have been reported compared to the variety of carotenoids reported, but it is

assumed that a substantial amount of bacterial carotenoids often are glycosylated [31, 124, 258]. They have great potential use as natural surfactants for the future chemical preparation of compounds useful as antimicrobial, antibacterial, and antitumor agents, or in industry [55].

Although best known for their antioxidant effect and as accessory light-harvesting components of photosynthetic systems, many carotenoids are also produced by non-photosynthetic heterotrophic bacteria and fungi [116, 120]. Carotenoid biosynthesis apparently has an early origin, before photosynthetic systems arose, when the levels of UV radiation were higher than today [43, 115, 189]. And it is therefore plausible that these pigments evolved originally to play a role in membrane stabilization and UV tolerance. Some microorganisms produce carotenoids with structural characteristics very different from those commonly found in plants, such as a higher number of carbon atoms, of conjugated double bounds, and of hydroxyl groups, which all contribute to their great antioxidant capacity [190].

In microalgae, a distinction can be made between primary and secondary carotenoids. Primary carotenoids, as lutein, function as accessory pigments in the photo systems, as structural components of light harvesting complexes in chloroplasts, and as photo protective agents, and are therefore essential for cell survival. Secondary carotenoids, such as astaxanthin, accumulate in large quantities in lipid bodies outside the chloroplasts, after subjecting cells to stress conditions [28, 107]. Such environmental stress conditions include high salt concentrations, high irradiation (sun light) and nitrogen deficiency.

Animals cannot synthesize carotenoids, but must obtain them in their diet and may employ them in various ways in the metabolism, e.g. β-carotene function as provitamin A that is converted to retinal and further to retinol (vitamin A) [187]. General proposed functions for carotenoid are to stimulate the immune system and play an important role in the prevention of degenerative diseases and cancer [45, 121, 185, 231]. However, studies examining carotenoid health effects have produced inconsistent results [234]. Individuals with higher carotenoid intake or serum carotenoid concentration (α-carotene, β-carotene, β-cryptoxanthin, lutein, lycopene, zeaxanthin) have lower risk of mortality [135, 162], lung cancer [97], prostate cancer [81], and coronary heart disease [172]. The antioxidant activity of these compounds may shift

into pro-oxidant activity by interventions of carotenoid supplementation specifically with β-carotene in tobacco smokers, facilitating the development of lung cancer [247] or no effects [1, 188]. This can be caused by the high concentration of individual carotenoids and possible pro-oxidant effects in settings of high oxidative stress. In addition, the positive effect attributed the carotenoids may reflect a dietary pattern associated with better health. For example, high lycopene concentrations suggest a diet rich in tomato products, which may also involve high intake of other fruits and vegetables.

1.5.1 Carotenoids as sun protecting agent

Sunlight is a continuous spectrum of electromagnetic radiation that is divided into three main wavelengths regions: ultraviolet (UV), visible, and infrared (IR). UV radiation comprises the wavelengths from 200 to 400 nm, while visible light ranges from 400 to 750 nm and IR above the visible (750 to 1000 nm). The ultraviolet spectrum is further divided into three sections, each with distinct biological effects: UVA (320-400 nm), UVB (280-320 nm), and UVC (200-280 nm). When sun rays hit the skin surface it consists primarily of infrared and visible radiation, with only a small portion being within the UV spectrum. The portion of UVA rays is 10 - 20 times higher than UVB rays. This means that even though the UVB rays induce skin damage more efficiently, UVA and visible rays reach the cells of the skin more frequently.

The damaging effects of UVB have been widely documented (reddening of the skin, skin cancers and premature ageing of the skin, damage the cornea). UVA penetrate deeper into the skin surface than UVB rays (Figure 1.3) and cause long-term damage such as premature wrinkling and photo aging. The UVA damage is believed to exacerbates the risk of melanoma and other tumors [125, 170]. UVA rays are not directly absorbed by DNA, but can have indirect harmful effects by forming radical oxygen species (ROS) that can react with cellular proteins and DNA (causing single strand breaks) [278].

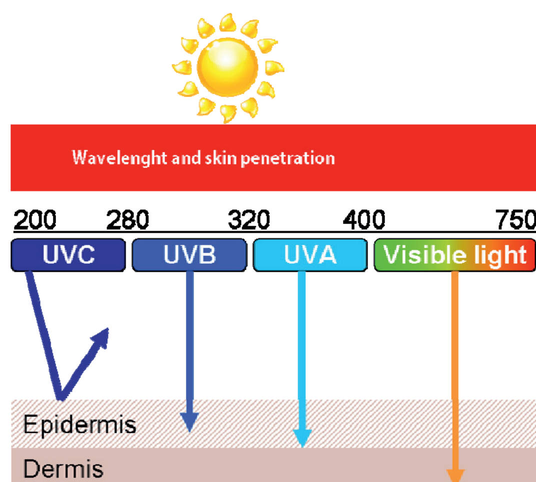

Figure 1.3. Penetration of solar radiation to the skin. The radiation penetrates the human skin with different efficiency, higher wavelengths penetrating deeper into the skin than lower. Adapted from [2].

With the growing awareness that UVA radiation causes significant damage, the need for broad spectrum (UVA/UVB) sunscreens has become obvious. Individuals using sunscreens normally expose themselves to the sun for much longer times than those without sun protection and thus will be more prone to melanoma when using UVB only sunscreen due to the prolonged exposure to UVA radiation [56]. To avoid this kind of "misunderstanding" the Food and Drug Administration (FDA), an agency of the United States Department of Health and Human Services, has imposed new labelling rules for sunscreens from July 2012 [70]. Products that do not pass the new "Broad Spectrum" testing requirements must include a warning that states: "*Skin Cancer/Skin Aging Alert: Spending time in the sun increases your risk of skin cancer and early skin aging. This product has been shown only to help prevent sunburn, not skin cancer or early skin aging*." This is also the case when the weather is cloudy; UVB rays are filtered, but not UVA. Health authorities therefore recommend both broad spectrum sunscreen and the use of sunglasses in cloudy weather as well as in sun exposure.

Within the sunscreen technology, there are very few agents that offer protection in the upper UVA spectrum and no acceptable compounds that protects against visible blue light spectrum, i.e. in the range 360-500 nm. Despite increasing awareness of the importance of broad-spectrum protection, studies show that commercially available sunscreens claiming to have good UVA protection do not protect sufficiently against UVA rays. Particularly, for the longer wavelength UVA radiation (370-400nm), the

available sun filters provide poor protection and very poor or no protection against wavelengths above 400 nm (see Figure 1.4). Most of the commercially available UV- and sun protecting compounds in skin creams are synthetic, and the search for natural compounds with equal or greater efficiency is increasing due to the consumer's preference for natural products.

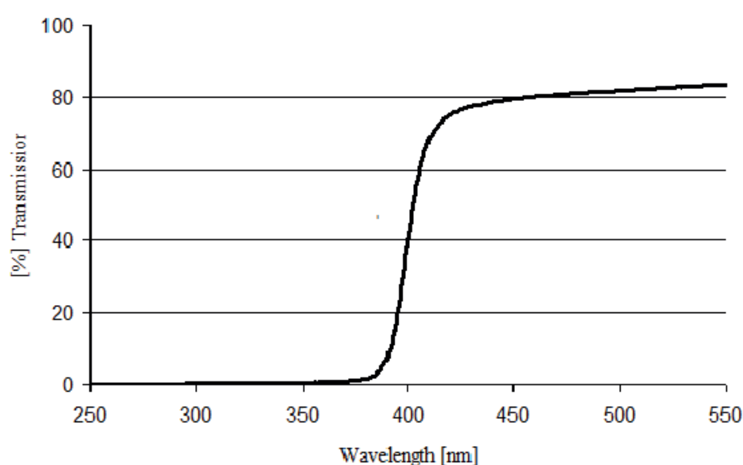

Figure 1.4. Transmission spectra of Sun Protection Factor 60 (SPF60). Adopted from [280].

Carotenoids have been proposed as a sun protecting agent based on the fact that they protect their producing organism against reactive oxygen species (ROS) and UV radiation from the sun by absorbing light in the 350-500 nm range [248]. This is within the range that should be added to obtain broad spectrum protection and therefore carotenoids absorbing in this range would provide complementary protection, increasing the protection of radiation above 400 nm. However, the solubility of carotenoids is a challenge and any solvent for use in cosmetics has to be approved by the FDA or health authorities in the respective country. An alternative to external application of carotenoids is internal uptake through the diet. Some tanning pills incorporate this pigment, which, when ingested, accumulates in the fat layer of the skin and produces a golden hue, simulating a tan and thereby proposed to protect the individual from the harmful effects of UV radiation. However, as discussed in section

1.5, the unequivocal benefit to humans of carotenoid supplementation has yet to be demonstrated.

Many carotenoids are already protected by patents for use as sun protecting agents; like astaxanthin, lycopene, β-carotene, phytoene, diadinoxanthin (http://www.faqs.org/patents/app/20080260662). But the vast diversity of available structures and the possibility offered by genetic engineering to construct new compounds with enhanced properties (e.g. absorption, antioxidant, solubility) still make them an attractive starting point for natural sun protection formulas [7]. In addition, there may still be many new structures awaiting discovery in the microbial community.

1.5.2 Isolation and characterization of carotenoids

A good extraction procedure should release all the carotenoids from the sample and bring them into solution without causing any change in them

The first step in carotenoid identification is to separate the carotenoids from the biomass (bacterial, plant, animal). The extraction protocol has to be optimized in each case depending on properties of the biomass and the carotenoids to be extracted. Only carotenoids from bacterial biomass will be discussed here. Carotenoids can be extracted from fresh bacterial biomass, which contain significant amounts of water, with water-miscible organic solvents. Lyophilized materials can be extracted with water-immiscible solvents. Water in the extract can also be removed by partition to hexane, petroleum ether, diethyl ether, or dichloromethane or mixtures of these solvents. A good extraction procedure should release all the carotenoids from the sample and bring them into solution without causing any change in them. Acetone is a better solvent for the least polar carotenes and methanol better for xanthophylls and therefore mixtures thereof are often used [246]. Multiple extractions are usually required to obtain a color-free cell pellet and complete extraction. An enzymatic treatment step to weaken the bacterial cell wall might be necessary, especially for Gram-positive bacteria, in order to release the majority of pigments. Physical methods like ultrasound, French press and bead homogenizer are also alternatives. Saponification is an effective means of removing unwanted lipids, which may interfere with the later chromatographic separation. However, saponification extends the total analysis time, and may cause artifact

formation and degradation of carotenoids as is also the case with partition [211]. In the end, the extraction efficiency depends on cell wall resistance, the solvent penetration power and the length of the extraction [109, 254].

The solvent that result from these extraction procedures must be removed from the solutions. Alternatively, solvents that are compatible with further analysis can be reduced to obtain desired volume or concentration. Aqueous solutions are lyophilized, while organic solvent mixtures are dried using rotary evaporators. In the latter case, solvents with low boiling point have to be chosen to avoid prolonged heating. Carotenoids should then be stored under nitrogen atmosphere at -80 °C if not analyzed immediately.

UV-Vis and spectral fine structure serves as a basis for carotenoid identification and quantification

The conjugated double-bond system constitutes the light-absorbing chromophore that gives carotenoids their attractive color and provides the UV and visible absorption spectrum (UV-Vis) that serves as a basis for their identification and quantification. The wavelength of maximum absorption (λmax) and the shape of the spectrum (spectral fine structure) are characteristic of the chromophore. Most carotenoids absorb maximally at three wavelengths, resulting in three-peak spectra (Figure 1.5 A). To give an idea of the spectral fine structure and to easier compare different spectra, the %III/II can be presented, along with the λmax values. The %III/II is the ratio of the height of the longest-wavelength absorption peak, designated III, and that of the middle absorption peak, designated II, taking the minimum between the two peaks as baseline, multiplied by 100 [33]. For conjugated ketocarotenoids, such as canthaxanthin and echinenone, the spectrum consists of a broad single maximum, having no defined fine structure, thus %III/II is 0 (Figure 1.5 B).

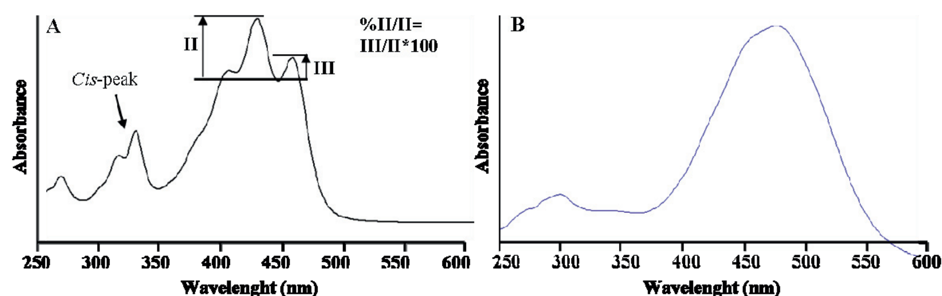

Figure 1.5. The UV-Vis spectre of a carotenoid with a distinct degree of spectral fine structure (A), and no fine structure (B). The calculation of % III/II for a carotenoid is inserted in A (adopted from [211]).

At least 7 conjugated double bonds are needed for a carotenoid to have perceptible color [210]. The greater the number of conjugated double bonds, the higher the λ_{max} values. Thus, the most unsaturated acyclic carotenoid lycopene, with 11 conjugated double bonds, is red and absorbs at the longest wavelengths (λ_{max} at 448, 474, and 505 nm in acetone). Several factors influence on the spectral fine-structure and absorbance wavelengths, like the chromophore, cyclization, steric hindrance (which can lead to hypsochromic shift, i.e. shorter wavelength), and placement of the carbonyl group.

Most carotenoids occur naturally in the all-*trans* form, but can easily be isomerized to mono-*cis* or poly-*cis* upon exposure to high temperatures, light, oxygen, acids, catalyst and metal ions, with a consequent change in light absorbing properties. *Cis*-isomerization occurs frequently of a chromophore's double bond and causes a slight hypochromic effect (lowering of absorbance intensity), small hypsochromic shift (usually 2 to 6 nm for mono-*cis*), a less pronounced spectral fine structure and accompanied by the appearance of a distinct *cis* peak by ~140 nm lower than the longest wavelength absorption [32]. The intensity of the *cis* peak is greater as the *cis* double bond is nearer the centre of the molecule. Thus, the 15-*cis* isomer, in which the *cis* double bond is in the centre of the molecule, has an intense *cis* peak. The 5,6-monoepoxide and 5,6,5′,6′-diepoxides of cyclic carotenoids, having lost one and two ring double bonds, respectively, absorb maximally at wavelengths some 5 and 10 nm lower and are lighter coloured than the parent compounds. These changes can easily be detected when analyzing their UV-Vis spectre. Many more examples of what effects the spectral fine structure and absorbance values can be found in [210].

The absorption spectra of carotenoids are markedly solvent dependent. The λ_{max} values relative to hexane, petroleum ether, diethyl ether, methanol, ethanol, and acetonitrile are practically the same but increases by 2–6 nm in acetone, 10–20 nm in chloroform, 10–20 nm in dichloromethane, and 18–24 nm in toluene [33]. UV-Vis absorption spectra obtained with diode array detector in HPLC systems (Figure 1.5) are aquired in mixed solvents and it is important to account for the influence of the solvent on the spectra properties.

Calculation of the carotenoid concentration

The absorption coefficient A1%1cm of a carotenoid (absorbance at a given wavelength of a 1% solution in a spectrophotometer cuvette with a 1-cm light path) used in the calculation of the concentration also varies significantly in different solvents. Carotenoids in solution obey the Beer-Lambert law, i.e. their absorbance is directly proportional to the concentration. Thus, carotenoids are quantified spectrophotometrically. The quantification, however, depends on the availability of accurate absorption coefficients, which are difficult to obtain. The procedure normally involves weighing a small amount of the carotenoid, typically 1 to 2 mg, with an accuracy of ±0.001 mg [33]. This requires an accurate and sensitive balance and the carotenoid should be free from contaminants, including residual solvent. Moreover, complete dissolution of the carotenoids in the desired solvent can be difficult. Thus, some published values may have significant level of error or uncertainty [33]. The concentration of each identified carotenoid can be calculated according to the following formula:

$$x\ (\mu g) = \frac{A \cdot y\ (mL) \cdot 10^6}{A_{1cm}^{1\%} \cdot 100}$$

where x is the weight of the carotenoid, y is the volume of the solution that gives an absorbance of A at a specified wavelength, and A1%1cm is the absorption coefficient of the carotenoid in the solvent used. When no absorption coefficient is available, an

accepted approximation is to use the molar absorption of a "similar" chromophore, which will not be accurate but sufficient for research purposes. In addition, the importance in carotenoid research lies more in the relative concentration of the total carotenoids and fold increase (or decrease) more than absolute concentration.

HPLC and MS for carotenoid identification

Heat labile carotenoids cannot be analyzed by GC and GC–MS. The most widely used method today is reversed phased HPLC equipped with DAD and MS detection (LC–DAD-MS). The reproducibility and high sensitivity provide reliable analytical data, and the reasonably short analysis time minimizes the isomerization and decomposition of these sensitive compounds [29]. The DAD allows the UV-Vis spectrum of each component to be determined on line and information about the mass of individual carotenoids can be obtained through MS. Atmospheric pressure chemical ionization (APCI) is an ideal method of ionization for low- to medium-polar compounds, which include carotenoids and related compounds [207, 209]. Figure 1.6 exemplifies an LC-DAD-MS analysis of carotenoids in a mixture. For protocols the reader is referred to the methods section in current research papers on carotenoid identification, *e.g.* [154, 176, 178, 206, 246] and further information on LC-DAD-MS analysis can be found in Paper I and IV.

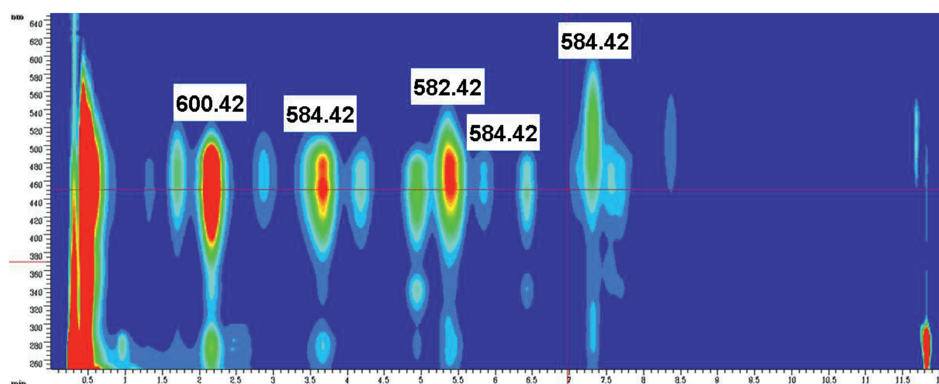

Figure. 1.6 LC-DAD-MS analysis represented by isoabsorbance plot of a methanol extract from a pigmented marine bacterial isolate. An isoabsorbance plot is a 3-D graph of time, absorbance and intensity in a 2-D window by the representation of intensity by color. Identified masses from MS analysis are given in boxes.

A given mass is far from conclusive as the majority of the carotenoids have a common C_{40} skeleton with different levels of hydroxylation and cyclization. Thus, there will be many different carotenoids with identical mass. From the masses given in Figure 1.7, possible molecular formulas and possible identification can be retrieved by using the online database DNP (see Table 1.1). DNP is the most comprehensive source of natural product information available. The results from the search on these particular molecular masses are given in Table 1.3. Candidate identities can then be removed from the list if their UV/Vis profile does not fit.

Table 1.3 Examples of molecular masses (m/z) values obtained with MS performed on a methanol extract of a pigmented marine bacterium. The carotenoid formula is calculated and candidate carotenoid identified by using the online database Dictionary of Natural Products (DNP). Optical stereoisomers are omitted from the list.

m/z	Carotenoid formula	Hits in DNP	Examples
582.41	$C_{40}H_{54}O_3$	14	Flexixanthin, α-Doredexanthin
584.42	$C_{40}H_{56}O_3$	19	Myxol, Nostoxanthin, 2'-Deoxy
596.39	$C_{40}H_{52}O_4$	10	Astaxanthin, Phillipsiaxanthin
600.42	$C_{40}H_{56}O_4$	27	Nostoxanthin, Violaxanthin

Thin Layer Chromatography (TLC)

TLC is widely used for food and pharmaceutical analyses because it is rapid, effective, and relatively inexpensive [289]. Furthermore, the TLC method does not necessarily require any instrumentation and the determination can be performed in the field, unlike HPLC. Although mostly replaced by HPLC for carotenoid analysis, it continues to be used for validation and complimentary analysis [113]. Preparative TLC as well as preparative HPLC can be used for isolation of pure carotenoids.

Nuclear Magnetic Resonance (NMR)

Once a carotenoid is isolated from the mixture and purified, it can be subjected to structure elucidation. The key technique for this is NMR, specifically a series of two-dimensional experiments (COSY, HSQC, HMBC, and NOESY) that makes it possible to establish the connectivity of all hydrogen and carbon atoms in a molecule. Although larger amounts of sample are needed than for MS, NMR is non-destructive, and with

modern instruments, good data may be obtained from samples of less than a milligram. It has therefore become widely used in carotenoid identification as LC-DAD-MS and chemical tests (see below) often are inconclusive. NMR can also be directly coupled to LC and yield 1-D and 2-D NMR spectra for the components separated by HPLC [26, 265]. The detection limit depends heavily on the NMR mode; ~100 ng in stopped-flow and loop storage mode and > 10 µg in continuous or onflow mode [5].

Chemical derivatization of functional groups as aid in structural elucidation

Xanthophylls undergo group chemical reactions that can serve as simple chemical tests for the identification of carotenoids. Many of the chemical reactions, in extensive use in the late 1960s and early 1970s, have now been replaced by MS and NMR. However, some reactions remain useful and can be performed quickly and require only a small amount of the test carotenoid, and are amenable to rapid monitoring by UV or visible spectrometry, or HPLC. The most common tests are summarized in Table 1.4.

Table 1.4. Summary of chemical reactions used as a diagnostic tool for carotenoid identification [35].

Functional group	Reaction	Catalyst	Observed change
Primary and secondary hydroxyl group	Acetylation	Acetic acid in pyridine	Increase in retention time[1)]
Allylic hydroxyls	Methylation	Acidic methanol	Increase in retention time[1)]
Epoxy group in 5,6 or 5',6' position	Conversion to furanoid derivatives	Acid	Hypsochromic shift, 20-25 or 50 nm
Ketocarotenoids and apocarotenals	Reduction	$LiAlH_4$ or $NaBH_4$	Conversion to three-maxima spectra

1) HPLC. The extent of the increase depends on the number of hydroxysubstituents

To identify a carotenoid, these minimum criteria should be fulfilled to avoid misidentification [197, 225];

• the UV/Vis absorption spectrum (λ_{max} and fine structure) in at least two different solvents are in agreement with the chromophore suggested

• a mass spectrum should be obtained, which allows at least confirmation of the molecular mass

• chromatographic properties are identical in two systems, preferably TLC and HPLC and co-chromatography with an authentic sample should be demonstrated

The last criterion is difficult to fulfil if no authentic sample is available and the identity of the carotenoid can then only be proposed. There are more than 750 known carotenoids and many of these will have the same chromophore and mass. In addition, there are potential undiscovered carotenoids. The carotenoid concentrations might be very low. Typically, one to three principal carotenoids are present, with an additional number of carotenoids at low or trace levels. These minor carotenoids can be intermediates in the carotenogenesis or isomerized or oxidized carotenoids, reactions that can easily occur during analysis (discussed under UV-Vis and spectral fine structure) [127].

1.5.3 Carotenoid genes and biosynthetic pathways in bacteria

Carotenoids belong to the tetraterpene group of the isoprenoids. Other isoprenoid groups are monoterpenes (e.g. menthol), sesquiterpenes (e.g. artemisinin), triterpenes (e.g. squalene), diterpenes (e.g. taxol), and polyterpenes (e.g. rubber). The biosynthesis of carotenoids starts with isopentenyl-diphosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) formation, the general precursors of all isoprenoids. These five-carbon (C_5) building units can be produced in two independent pathways: the mevalonate (MVA) pathway [151], and the more recently discovered 1-deoxy-D-xylose-5-phosphate (DOXP) pathway (also called the MEP pathway or the non-mevalonate pathway) [130, 213]. The two pathways are illustrated in Figure 1.7. In most bacteria, isoprene is produced via the DOXP pathway proceeding from pyruvate and D-glyceraldehyde-3-phosphate (G3P), whereas *Archaea*, eukaryotes with the exception of photosynthetic eukaryotes, and some bacteria utilize the mevalonate pathway, beginning with the synthesis of acetoacetyl-CoA from two molecules of acetyl-CoA. Some Actinomycetes possess both pathways [50-52].

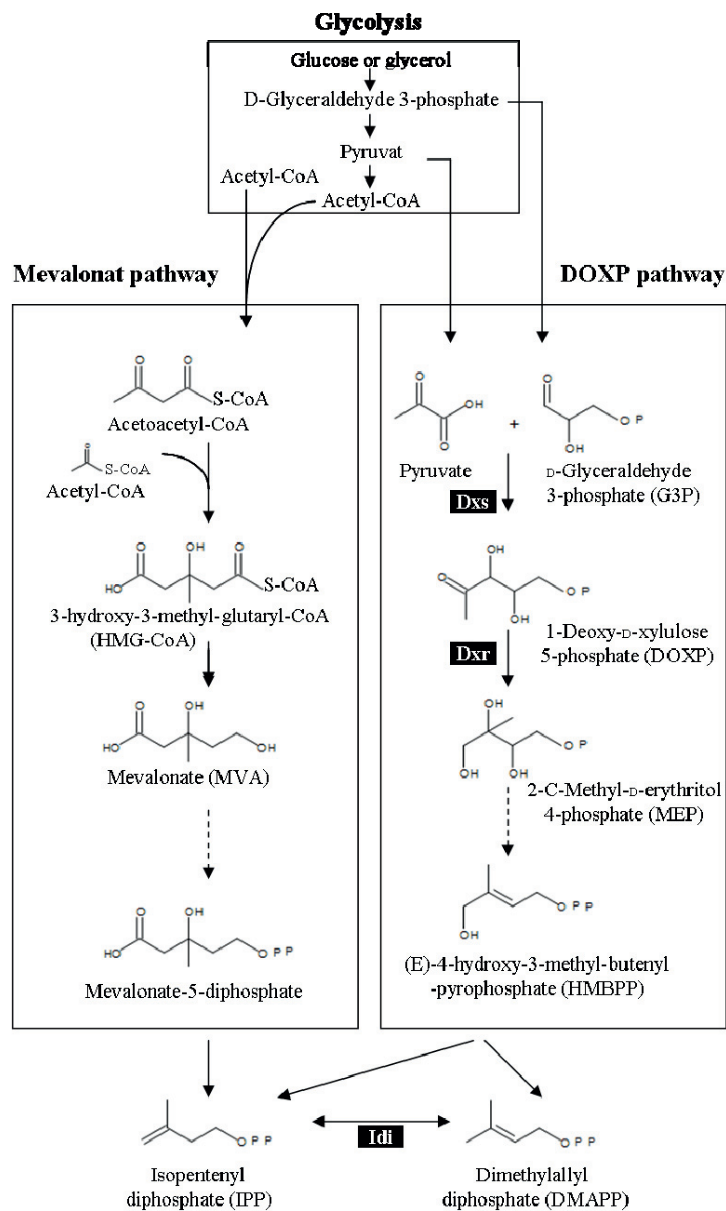

Figure 1.7. The mevalonat and DOXP pathway leading to the common C_5 precursors for isoprenoid biosynthesis. Key regulatory enzymes in the pathway are given in black boxes. In addition, the precursors for these pathways via the glycolysis are shown.

The carotenoid biosynthesis proceeds through a conserved central pathway starting with the condensation of the isomers DMAPP and IPP forming geranyl pyrophosphate (GPP, C_{10}) and further condensation with two IPP units to form geranylgeranyl pyrophosphate (GGPP, C_{20}) and finally the colorless phytoene is formed by condensation of two GGPP molecules (Figure 1.8). In total eight IPP units are joined to form phytoene, which is the first molecule defined as a carotenoid and the starting point for all C_{40}, C_{50} and C_{35} carotenoids [240]. In the synthesis of C_{30} carotenoids, two FPP molecules are condensed as depicted in Figure. 1.8.

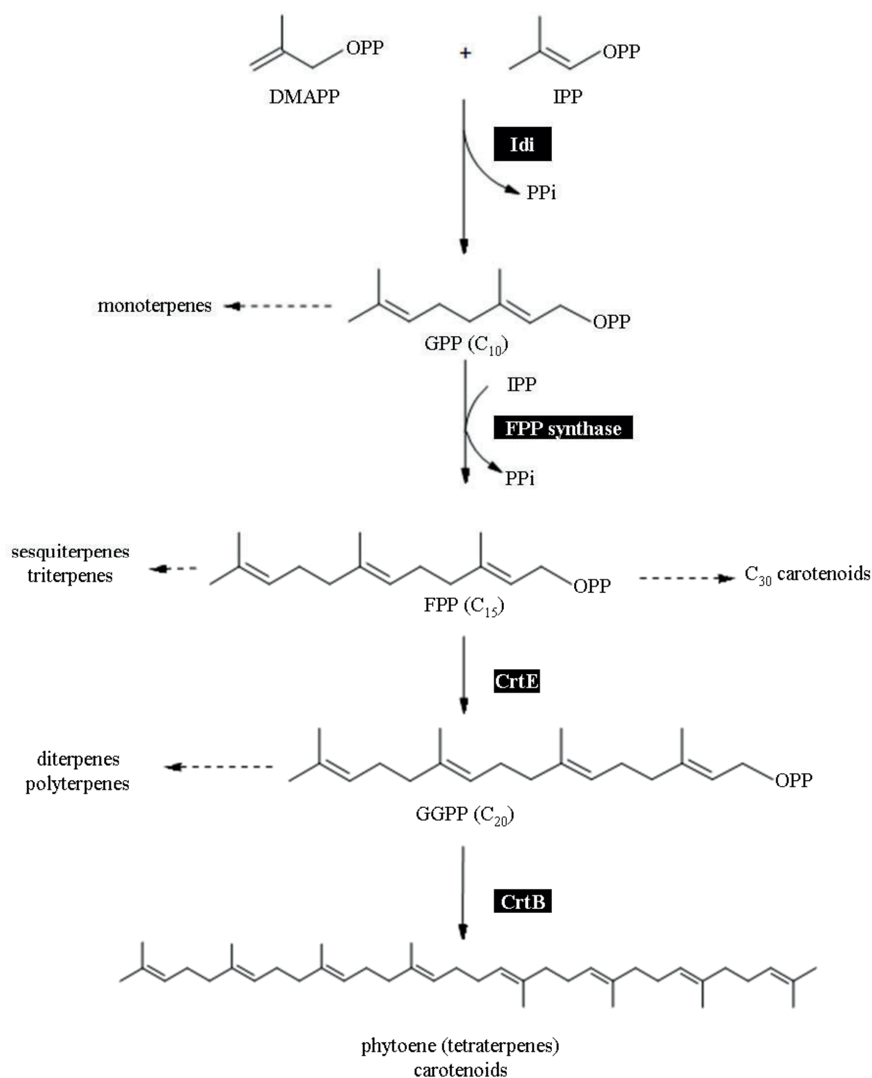

Figure 1.8. The biosynthesis of phytoene, from the C_5 building units dimethylallyl diphosphate (DMAPP) and isopentenyl diphosphate (IPP) via geranyl pyrophosphate (GPP), farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP). The enzymes are given in black boxes. GPP, FPP, and GGPP lie at multiple branch points in the isoprenoid pathway and are substrates for many enzymes, responsible for generating the diverse carbon skeletons for the further synthesis of the thousands of mono-, sesqui-, di-, tri- and polyterpenes found in nature.

The most important structural modification of carotenoids is desaturation [221]. In most bacteria, phytoene is desaturated by the phytoene desaturase (CrtI) through four consecutive steps to produce the red pigment lycopene. Lycopene and other carotenoids formed early in a pathway, are further modified depending on the enzymes present in the producing organism. The result is an extensive pathway branching (and sub-branching) illustrated by some examples in Figure 1.9 [100, 124, 166]. Depending on the enzymes present usually only one to five different carotenoids are produced in one organism [270].

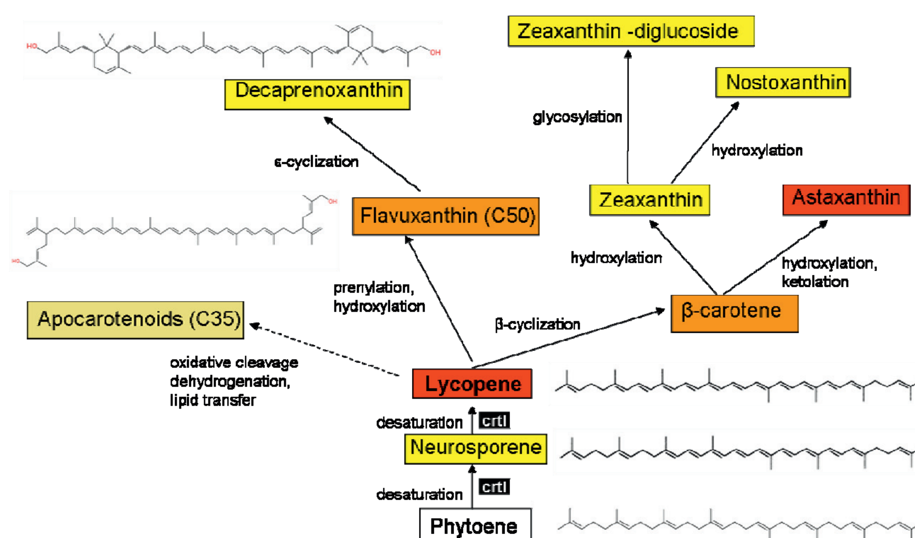

Figure 1.9. Branching of carotenoids, starting from the colorless C_{40} backbone phytoene. These backbones are desaturated, cyclized, oxidized glycosylated and otherwise modified by downstream enzymes in various species-specific combinations. Shown are several common types of enzymatic transformations that occur in natural carotenoid pathways. The color-coding reflects the color of the carotenoid. Structure formulas for some of the carotenoids are included to show the effect of desaturation, prenylation, hydroxylation and cyclization. Apocarotenoids are formed from oxidative cleavage of C_{40} xanthophylls [218].

Diprenylation (addition of two C_5 isoprene units) and hydroxylation of C_{40} carotenoids leads to C_{50} carotenoids. C_{50} carotenoids have been found in Gram-positive bacteria from the order *Actinomycetales* (species from the genera *Micrococcus*, *Halobacterium*,

Corynebacterium, Dietzia, and *Flavobacterium*) [14, 31, 122, 144, 217, 252, 260] in addition in the Gram-negative *Pseudomonas* strain [165].

C_{30} carotenoids are another minor group. The carotenoid pathways start from a different branching point in the isoprenoid synthesis; with the condensation of two molecules of FPP (Figure 1.8). They are found in various unrelated bacterial genera; *Methylobacterium rhodium*, *Streptococcus faecium, Staphylococcus aureus*, *Heliobacteria*, *Rubritella squalenifaciens, Planococcus maritimus*, *Halobacillus halophilus* and *Bacillus firmus* [117, 118, 159, 237, 238, 250, 257, 261]. In contrast, C_{35} carotenoids known as "apocarotenoids" are cleavage products of oxygenated C_{40} carotenoids (xanthophylls) and thus belong to the C_{40} family (Figure 1.9) [218]. Examples are neurosporaxanthin produced by the fungi *Neurospora* and the commercially valuable pigments bixin and saffran produced by plants [18].

1.5.4 Exploring the genes involved in carotenoid biosynthesis

Since Misawa and co-workers cloned the carotenoid biosynthesis gene cluster from *Pantoea ananatis* (previously *Erwinia uredovora*) in 1990 [167], many carotenoid pathways have been elucidated. Most bacteria have their carotenoid genes organized in a single gene cluster, while other carotenoid producing organisms, such as *Chlorobaculum* (previously *Chlorobium*) *tepidum* (green sulfur bacteria), cyanobacteria, algae and higher plants, have no such clusters and instead the genes are randomly distributed on the genome [101, 145]. Single genes can also be localized outside the rest of the carotenogenesis gene cluster as is the case with β-carotene hydroxylase gene (*crtZ*) involved in the synthesis of nostoxanthin in *Sphingomonas elodea* [292] or glycosyl transferases (*crtX*) in *Enterobacteriaceae* strains [232]. Recently, Wendisch and co-workers [94] have found a second carotenoid gene cluster in *Corynebacterium glutamicum,* both containing a functional phytoene synthase gene (*crtB*).

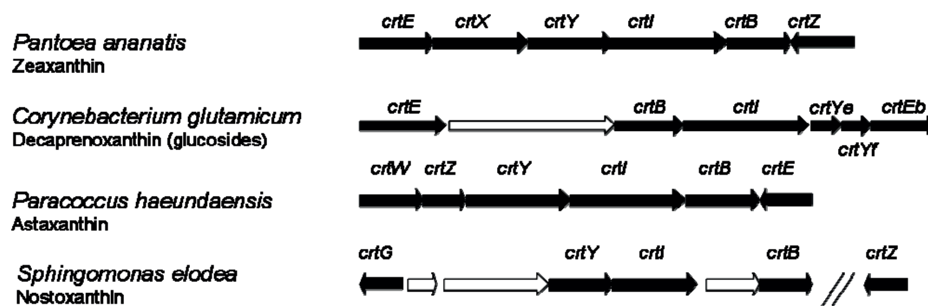

Figure 1.10. Illustration of the carotenoid biosynthetic gene cluster for *P.ananatis*, *C.glutamicum* [124], *P. marcusii* [138] and *S.elodea*. The genes are presented as arrows pointing in the direction of their transcriptions. Genes indicated in white are suggested not to be involved in carotenoid biosynthesis.

Cosmids are cloning vectors able to contain ~30-45 kb DNA [88] and are very useful for studying the gene organization in bacteria. All known carotenoid gene clusters are smaller than 30 kb and pigmented colonies are expected to be observed when constructing a cosmid library from carotenoid producing bacteria. Misawa and co-workers reported pigmented colonies in a ratio of one to several hundred from their studies in elucidating the carotenoid biosynthetic pathway [167, 168]. The pigmented cosmid clones can then be sequenced, and the carotenoid synthesis gene clusters be determined from sequence assembly. Alternatively, a specific carotenoid DNA probe can be amplified from genomic DNA based on degenerate primers and used for hybridization experiments with the cosmid library. Well-conserved domains in the N-terminal region and C-terminal region in the *crtI* encoding phytoene desaturase is the first choice when designing primers due to higher homology among different bacteria compared with the genes encoding for GGPP synthase (*crtE*) or Phytoene synthase (*crtB*), see Figure 1.8. However, this approach is challenging because in contrast to genes from plants, bacterial *crt* genes are not highly conserved and do not even hybridize to sequences of closely related species [123]. The approach with degenerate primers can also be applied to metagenome studies when searching for carotenoid genes or any other genes with prior knowledge of the target gene.

A third alternative is DNA sequencing of the entire bacterial genome and subsequent genome mining. In terms of costs and complexity it is getting a more and more realistic first choice [262]. As of today (December 2012), there are 3329 complete

genome sequences of bacteria listed on the online NCBI genome database (http://www.ncbi.nlm.nih.gov/sites/genome). With the entire genomic sequence available, potential carotenogenesis genes can easily be identified by sequence alignments and gene annotation. This can also be generally applied to the field of microbial natural product research; the increasing availability of genome sequences provides an enormous potential for the discovery of new natural compounds. The examination of biosynthetic gene clusters accessible from sequence information can further prevent rediscovery of known compounds.

Knowledge about the function of the genes in the pathway makes it possible to construct new carotenoids with potentially improved properties compared to existing natural carotenoids (i.e. enhanced antioxidant activity) [199, 227, 270]. Carotenoid enzymes from different organisms have been combined to generate functional pathways in heterologous hosts this proves that the formation of a specific enzyme complex is not a prerequisite for carotenoid biosynthesis, instead they have been proven to work independently [180, 270].

1.5.5 Industrial carotenoid production

Carotenoids are as group valuable molecules for the pharmaceutical, chemical, food and feed industries, not only because they can act as vitamin A precursors, but also for their coloring, antioxidant and possible tumor-inhibiting activity. The global marked value for carotenoids was estimated to US$ 1.2 billion in 2010 and is expected to reach 1.3 billion by 2017. The estimates are based on information from 82 companies worldwide [82]. Currently the carotenoids on the market are mainly used as food colorants and pigments in feed. Rising awareness about the benefits offered by various carotenoids is driving marked growth also for carotenoid dietary supplements, both synthetic and natural.

The feed sector represents the largest end use segment due to widespread use of astaxanthin (salmon) and canthaxanthin (poultry and salmon industry). The annual demand for astaxanthin worldwide in 2010 was 110-120 tons and is predicted to grow to 150-160 tons in 2020 [82]. β-carotene is the most widely used food colorant and is principally used in yellow fats (butter, margarines, low fat spreads etc), but also soft drinks, confectionery and bakery products [60]. β-carotene was first marketed in 1954

and today the marked demand is thought to exceed 500 tonnes [82, 85]. Carotenoids are alternatives to chemically synthesized food coloration; in particular, β-carotene for yellow-orange color shades and lutein for yellow-gold color replacements, and the consumer demand drives the development toward more natural additives. Between 2006 and 2009 Nestlé replaced four undesired colors for food in Smarties with a combination of colors including β-carotene, copper chlorophyll, riboflavin, and curcumin [266].

Most of the carotenoids on the market are nature-identical carotenoids produced by chemical synthesis, but a few are produced in biological systems although these are not always cost competitive with chemical synthesis [142, 164, 177]. These are the algae *Dunaliella salina (*β-Carotene) and *Haematococcus pluvialis* (astaxanthin), the fungus *Blakeslea trispora (*β-Carotene) and the yeast *Phaffia rhodozyma* (astaxanthin). Natural sources of other carotenoids are plants and vegetables, e.g. *Tagetes erecta* (lutein) [194, 198], red fruit of *Capsicum annuum* (capsanthin) [271], and tomato (lycopene) [119]. Compared to the extraction from vegetables or chemical synthesis, the microbial production of carotenoids is of paramount interest. This is mainly due to the problems of seasonal and geographic variability in the production of carotenoids of plant origin and consumer demand for high quality and "natural" food additives. For instance pathways for the biosynthesis of saffron, the worlds most expensive spice ($40–50 per gram), have recently been identified by the Swiss firm Evolva, which anticipates commercial microbial production in 2015 or '16 [27]. Today's production requires more than 75,000 flowers (*Crocus sativus*) to yield just 0.45 kilo of saffron spice [163]. The potential of utilizing cheaper carbon sources such as waste from the agricultural industry has further increased the probability of a cost competitive product compared with chemical synthesis [193]. For these reasons, the production of carotenoids in microbial hosts has been and still is the focus of extensive research [6, 9, 10, 17, 25, 30, 69, 129, 156, 222, 228, 285]

1.5.6 Engineering microbial cell factories for industrial carotenoid production

In order for microbial carotenoid production to become cost competitive, the production process must be improved since titers produced by wild strains can never compete with the synthetically produced carotenoids. The first step in improving the carotenoid

production is optimization of various environmental and cultural conditions. In particular, the effect of light, temperature and chemical compounds in the media are to be optimized for maximum carotenoid production. E.g. the carbon to nitrogen ratio in the medium was found to increase the canthaxanthin production 5-fold in a *Brevibacterium* strain, resulting in a 700 ppm production level (0.07%) [179]. Improvements in the range 20 – 75 fold have also been achieved when optimizing the various cultural conditions [25]. However, when the original titers are in the ppm range, the impressive fold increases are still not sufficient for commercial production. For comparison 1.5 - 4 % astaxanthin is obtained in the unicellular green algae *Haematococcus pluvialis* [17, 153] and β-carotene in *Dunaliella salina* can reach an impressive 10% of its dry weight [201]. The main challenge with carotenoid production in algae is the slow growth rate (0.2 – 0.7 day^{-1}) [255].

To further improve the production yield, several microorganisms have been explored as hosts for heterologous carotenoid production, e.g. the fungi *Saccharomyces cerevisiae* [131, 275] and *Candida utilis* [236], and the bacteria *Zymomonas mobilis, Agrobacterium tumefaciens* [169], and *Escherichia coli* [51, 253]. *E. coli* grows fast, can utilize low-cost carbon substrates, is easy to scale-up and genetic tools are well established. Therefore, it is a very convenient host for heterologous carotenoid production. The existence of two pathways for IPP biosynthesis allows for consideration of complementary approaches for optimization of precursor supply in the production host (see Figure 1.7). One approach is to alter the metabolic flux and regulation of the native pathway; the alternative is to introduce the heterologous pathway to supplement the native pathway.

E. coli has to cope with the drain of prenyl pyrophosphates when carotenogenesis is established, which again can limit isoprenoid production yields. Overexpression of limiting enzymes of the native DOXP pathway and subsequent reactions can increase the supply of the C_5PP "starter unit" DMAPP (Black boxes in Figure 1.7). Overexpression of *idi* has been shown to increase carotenoid titers by approximately an order of magnitude [277]. *Dxs* and *dxr* and are also among the genes that have been transformed in *E.coli* under a strong promoter to enhance the supply of precursors [161]. Because GGPP is the direct substrate for the formation of the first carotenoid in the pathway and its level is comparably low in *E. coli*, high expression

levels of GGPP synthase (CrtE) are very important for carotenogenesis (Figure 1.8). Another bottleneck for carotenoid biosynthesis in *E. coli* was relieved in the pathway by overexpressing the gene which encodes phosphoenolpyruvate synthase (*pps*), a pyruvate consuming enzyme, indicating that the pools of G3P and pyruvate, which both are substrates of 1-deoxy-D-xylulose 5-phosphate synthase (Dxs) (Figure 1.7), have to be more balanced in the direction of G3P [69].

The introduction of heterologous MVA pathway (see Figure 1.7) genes into *E.coli* has been shown to improve the productivity of carotenoids [286]. It has also been showed that the production levels of carotenoids produced were significantly increased with the addition of exogenous MVA, such as $_{D}$-mevalonolactone (D-mevalonic acid lactone; D-MVL) in the culture medium. However, addition of mevalonate as a substrate makes it a costly process and efforts have been made to utilize the cheaper substrates, like acetoacetate, from earlier steps in the MVA pathway [91].

When the precursor pool is balanced in the direction IPP, precursor supply can no longer be the limiting factor. Instead, carotenoid storage in *E. coli* seems to be the next major bottleneck. Enhanced carotenoid production can cause membrane overload with loss of functionality [161]. Sandmann [222] suggests that future activities should focus on extending the carotenoid storage capacity by genetic modification of the density of membranes in *E. coli* cells or by establishing plastoglobuli-like structures as all the lipophilic carotenoids are sequestered in the cell membranes.

Bacterial plasmids are used as vectors to carry the gene of interest to be overexpressed. Generally, a high copy number leads to high productivity, but it also tends to impose metabolic burdens on cells and thereby lowering the yield and the production reproducibility of recombinant molecules. In addition, it requires a high selection pressure. Plasmid-free cells lead to losses in the entire product recovery and decrease the profitability of the whole process. Antibiotic-resistant genes are the most common selectable markers used in fermentation to prevent plasmid-free cells from overgrowing the culture and is widely used in research. Antibiotics, however, are expensive compounds and they can contaminate the biomass or final product. The regulatory approvals for many commercial fermentation products are more favorable if antibiotic resistance genes are not present in the production strains [235]. In the industry therefore, non-selective conditions are desired, which requires high plasmid stability to

avoid loss of production. Increased plasmid stability is therefore an aim towards industrial scale biotechnological production [196]. The metabolic burden can be drastically lowered by utilizing low-copy plasmids with a tightly regulated inducible promotor [105, 128]. In addition, these plasmids are more stably maintained in the cells. In the non-induced state only limited basal expression should occur and high level expression should be induced when sufficient cell-growth is reached. Leaky expression can cause metabolic burdens on the cells during the growth period by diverting the carbon and energy source to premature protein formation [269].

An alternative is to construct vectors that can be selected for by other acceptable mechanisms. These can be post-segregational killing by which plasmids are stably maintained by expressing a gene product that would be toxic to cells becoming plasmid-free upon division [78, 83] or essential gene complementation where the plasmid introduced codes for an essential gene for which the heterologous host has a defect or inhibited expression of [59].

Plasmids may be the best choice for the cloning and short-term expression of recombinant genes, in particular for the maximum overproduction of a given protein. However, a too strong gene expression may be unfavourable for long-term productivity [224]. Kim and co-workers [287] observed that a high expression of lycopene biosynthetic genes in *E. coli* leads to a decrease in growth and lycopene production. On the other hand, a low enzyme activity of a heterologous downstream pathway can result in a reduced product yield or in an accumulation of pathway intermediates [141].

An alternative to genes on plasmids is stable integration of heterologous genes into the host's chromosome. This alleviates the use of antibiotics that are required to exert selective pressure for plasmid maintenance during fermentation. In addition it eliminates the metabolic burden effects of multiple plasmids, the structural instability and segregational instability [74, 140]. However, time and effort of the more complex cloning procedure have to be considered in relation to the advantages of plasmid-free systems in upstream-processing.

2 Objectives for the thesis

Prior to the present study, microorganisms from the marine surface microlayer (SML) had been collected as part of a larger project in marine bioprospecting. The SML is exposed to much higher radiation than the under laying water, and therefore selective pressure is suggested to have enriched the SML for bacteria with defence mechanism for UV radiation, like production of carotenoids absorbing light in the range 375 – 450 nm. One aim with the current study was to identify pigments, preferably novel, from this collection for potential commercial interest together with our industrial partner Promar AS.

After an initial screen for pigments, the selected pigment(s) and producer strain(s) should be characterized and cultivation conditions for pigment production in the wild type optimized. However, only microalgae are known to be able to produce and store high amounts of carotenoids in lipid globules (up to 10%) whereas bacteria have a much lower storage capacity in their membranes. To increase the production of the desired carotenoid it is crucial to identify the biosynthetic pathway. The genes involved in the carotenoid synthesis can then be overexpressed in *E.coli,* a well studied and attractive host organism, and potential higher production achieved. A further sub-goal for this study was strain optimization and development of genetic tools for increased carotenoid production in *E.coli* or wild type host.

In addition, characterization of the bacterial collection to improve the value for future bioprospecting studies was an aim. In the first part of this project, we experienced that a lot of effort was put into analyzing replicas. Therefore, developing more rational tools for exploration of the bacterial collection with a special focus on dereplication was the focus for the last part of the study.

3 Summary of results and discussion

The results of this PhD work are described in Papers I-III and the following chapters are summarizing the major findings as well as discussing the results in a broader context and including some unpublished results.

3.1 Screening of the bacterial collection for carotenoids

The pigmented bacterial collection consisted of ~2000 isolates and most of the pigments were presumed to be carotenoids as they are the largest pigment group in marine heterotrophic bacteria (See Paper IV for review on pigmentation of Marine Heterotrophic Bacteria). Carotenoids are readily soluble in organic solvents, but the extraction protocol has to be optimized for the type and nature of the carotenoids present (xanthophylls, carotenes, hydrophobicity) and the properties of the biomass from which they are extracted from. After initial tests on ten different bacterial isolates, pure methanol was found to give the best overall extraction efficiency for this heterogeneous collection of bacterial isolates and carotenoids when compared with a mixture of methanol and acetone. A complete extraction was not the aim, because at this step the characterization of the isolates was primarily qualitative. A pre-treatment step with lysozyme was included in the extraction procedure for the isolates that were selected for detailed analysis, i.e. purification and structural elucidation of major carotenoids.

3.1.1 LC-TOF-MS analysis reveals high carotenoid diversity in the isolates (Paper I)

A high-resolution LC-MS protocol for analyzing a broad spectrum of pigments was designed. An additional aim was to provide important information for designing a high throughput protocol for screening a higher number of isolates later. An Agilent 1100 series HPLC system equipped with a diode array detector (DAD) that recorded UV-Vis spectra between 200 and 650 nm and Time of flight (TOF) mass spectrometer for high mass accuracy was used and operated in positive APCI mode. This enabled accurate mass determination with better than 3 ppm accuracy. Chromatographic separation was

performed with a reverse phase C18 4.6×150 mm column operated with flow of 1 ml/min. Mobile phases were methanol-water (80:20) in channel A and dichloromethane in channel B. The total run lasted 25 minutes.

Extracts from sixteen randomly chosen isolates cultivated in shake flasks were analyzed with the high resolution LC-DAD-TOF MS protocol. The number of pigments per isolate varied from one to ten with mostly three to five main abundant pigments plus several minor pigments. Figure 3.1 A shows a DAD isoabsorbance plot of a representative extract with three major and at least three minor pigments. The UV-Vis scans for the six largest peaks are shown in Fig. 1B. The chromatogram retention time (rt), measured masses, tentative molecular formula deduced from accurate mass determination and assumed C_{40}-carotenoid structure backbone, and spectral fine structure (%II/III) are inserted. The UV-VIS scans show that the extracted pigments all have the three-peak carotenoid profile. Peak 4 and 5 contain a distinct *cis*-peak (indicated by an arrow) and are putative isomers of peak 1 and 2, respectively as they also have identical masses. *Cis*-isomerization of the *trans*-form of carotenoids is likely to occur during extraction and sample processing, and a lower concentration of the *cis*-form is therefore most likely an artefact, and not a natural pigment in the producing organism [31, 171]. C*is*-isomerization leads to a hypsochromic shift in the UV-Vis absorbance properties, lower intensity and less pronounced fine structure in addition to the distinct *cis*-peak (section 1.5) [32]. The calculated %III/II as a measure for the spectral fine structure for each individual peak support the assumption of two main UV-Vis profiles including their *cis*-isomers; peak 1,3,4 (and 6) in one group and peak 2 and 5 in the other. The intensity of peak 6 is too low to give reliable values. The TOF MS was used to assign accurate molecular masses to each of the six peaks. Peaks with the same fine structure also have the same mass (except peak 6), which again supports the assumption that the pigments with the same DAD profile are isomers, i.e. *cis-trans* or Z/E of the same carotenoid. The mass 551.43 obtained for the peak 6 could possibly represent a loss of oxygen from the mass 567 (M-16) although this is not common [66].

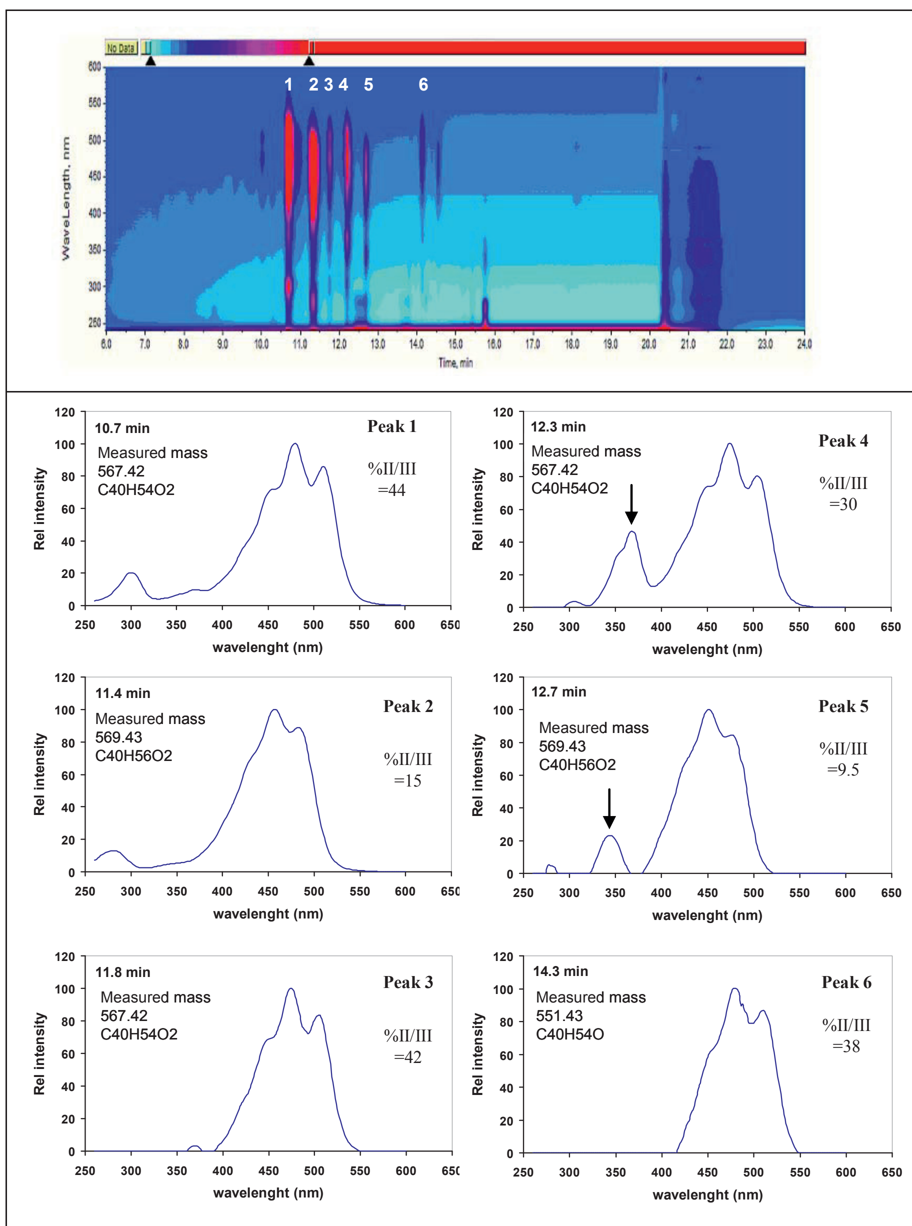

Figure 3.1. Example of a chromatographic scan presented as isoplot from 6 to 24 minutes. The measured mass, calculated molecular formula and %II/III as indicator of the spectral fine structure are inserted. All DAD scans show the typical three-peak-spectre characteristic for carotenoids. Peak 4 and 5 are carotenoids with pronounced *cis*-peaks (indicated by arrows). (Modified from Figure 1, Paper I.)

The high resolution analyses indicated large carotenoid diversity among the isolates in the culture collection, comprising both hydrophobic carotenoids (eluting late in the LC run) and more hydrophilic carotenoids (eluting rapidly in LC run), Figure 3.2. We obtained several masses from the LC-TOF analysis with carotenoid-like absorption spectra that did not give any hits in DNP and these are all potential novel and previously undiscovered pigments. Pigments with masses higher than 710 may be glycosylated carotenoids, i.e. carotenoids linked to a sugar by a glycosidic or an ester link. Other natural pigments that do not belong to the carotenoid family but have a similar terpenoid conjugated backbone structure and therefore also similar absorbance spectra, are also described in the literature (see paper IV). No further attempts were made to identify these unknown compounds.

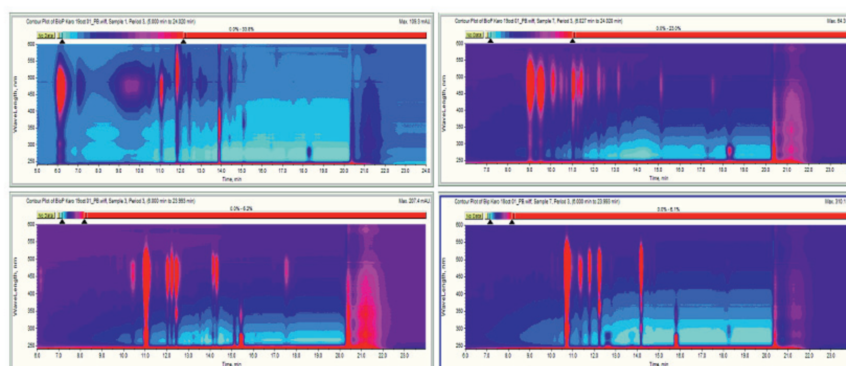

Figure 3.2 Diodearray isoabsorbance plots of four unique bacterial isolates from the culture collection. These plots visualize part of the large diversity among pigments and isolates. (Figure 2, Paper I.)

Tentative identification of the pigments was based on the UV-Vis profile (DAD) and molecular formula calculated from the accurate mass determination. Most of the pigments were found to be carotenoids. As discussed in the introduction chapter, matching UV-Vis profile and molecular mass are not enough to identify a carotenoid. However, by comparing the carotenoids with identical molecular mass from DNP and consulting the Carotenoids Handbook [34] for UV-Vis profiles, the number of candidate carotenoids can be narrowed down considerably. The main purpose of this study was to look for unique pigments and the accurate mass and DAD profile data combined with

the retention time index makes it possible to discriminate between different carotenoids in the various extracts.

3.1.2 High throughput LC-MS analysis enables quick characterization of carotenoids (Paper I)

The chromatographic resolution may decrease with a shorter run period and carotenoids that are separated in a long analysis run can co-elute in a shorter run, but to increase throughput compromises with regard to chromatographic separation must be made. However, screening and initial characterization of isolates focus on the major carotenoids in the extracts, and these will dominate the chromatogram in a fast analysis, too. As seen from the example illustrated in Figure 3.1, many of the minor carotenoids are often isomers of the main carotenoids and therefore we do not expect to loose much information in a high throughput screen.

A high throughput LC-MS method was established with the aim to screen the bacterial isolates for abundance and diversity of carotenoids. In addition, our main focus was on carotenoids that absorbed in the UVA-Blue light area. An Agilent Single Quadrupole (SQ) SL mass spectrometer equipped with an Agilent 1100 series HPLC system was used. The mobile phases were the same as for the protocol in the initial screen and the carotenoids were eluted during a 4 minute run. The column was re-equilibrated with a 3 min post run giving a total run time of 7 min. A Zorbax rapid resolution cartridge RP C18 column with dimension 2.1×30 mm was used for the analyses.

The SQ MS was set up with Single Ion Monitoring (SIM) for 20 carotenoid masses for 90% of the cycle time and 10% of the cycle time was run in scan mode. The 20 carotenoid masses (M+H+) that were included in the SIM list were generated from analysis of the LC-TOF MS data on the sixteen isolates and a thorough analysis of all carotenoid entries in DNP. The SQ MS has only unit mass resolution, but this is precise enough to assign a carotenoid molecular formula since carotenoids have limited variation in chemical composition. With $C_{40}H_{56}$ (lycopene) as the starting point, the carotenoids can be added additional O, H and C atoms through hydroxylation, prenylation and glycosylation, i.e. molecules contain only the elements C, H, and O.

A total of 260 isolates were analyzed with the high throughput screening LC-MS method. They were chosen with a criterion of diversity and represented eight different sampling points (see map, Figure 3.3).

The number of pigments detected per isolate was as expected lower with the short run LC-MS method than the long run method (1-3 major pigments versus 3-5 major pigments). Almost all pigments (>95%) could be assigned a carotenoid mass from the SIM-list implying that most of the pigments probably belong to the chemical group carotenoids. All 260 chromatograms were examined for carotenoids with an UV-Vis absorbance profile covering the UVA-Blue range of the light spectre and strains with main carotenoid(s) (pigment) with λmax below 450 nm were chosen as candidate strains for further investigations. Figure 3.4 shows the UV-Vis spectra of two such carotenoids. The comparison with astaxanthin visualizes the downward shift in the UV-Vis absorbance profile of two carotenoids.

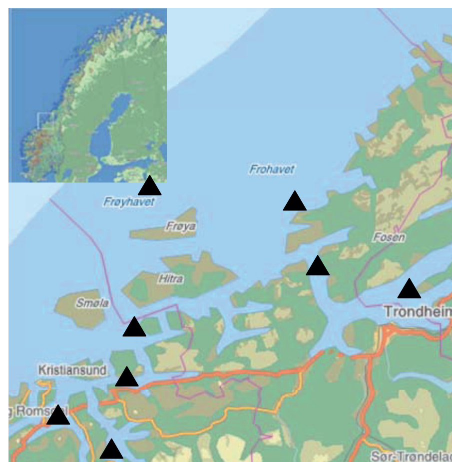

Figure 3.3 Map of the sampling locations along the mid part of the Norwegian coast. Samples were taken both in inner fjords and further off the coast.

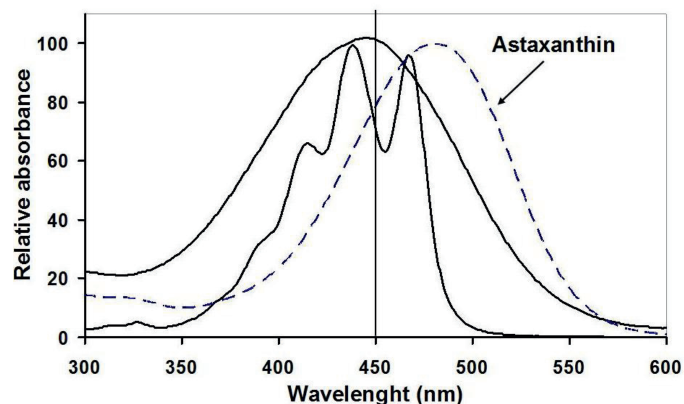

Figure 3.4. The UV-Vis absorbance spectre of two pigments absorbing in the UVA-Blue range. Astaxanthin is included for comparison. The inserted vertical line at 450 nm represent the screening criterion Organisms with a main carotenoid with λ_{max} below this value were chosen as candidates for further research (Figure 4. paper I)

3.1.3 Identification of six producer strains and carotenoid structural elucidation revealed potential novel strains and carotenoids with novel glycosylation pattern (Paper I)

Six strains containing main carotenoids with the desired UV-Vis properties were chosen for detailed analysis. The isolates were sent to NCIMB Ltd in Scotland for 16S rRNA analysis. Two strains were identified at the species level and the other four were identified at the genus level (Table2). This indicated that the bacterial collection also has great potential for finding novel strains. The six isolates were then cultivated in 1 liter fermentors to produce enough biomass for purification of carotenoids by preparative HPLC. Four isolates produced satisfactory amounts of carotenoids (measured in mg/L) and were selected for preparative HPLC analyses and subsequent structural elucidation using NMR. The results are given in Table 3.1.

Table 3.1. Results from identification of strains and carotenoids. (Modified from Table 3, Paper I).

Isolate	#Pigments/isolate	Carotenoids identified		
Leeuwenhoekiella sp	3	Zeaxanthin	9-*cis*-Zeaxanthin	13-*cis*-zeaxanthin
Spingomonas baekryungensis	5	4-ketonostoxanthin	Nostoxanthin	
Erythrobacter sp.	2	Nostoxanthin diglycoside	Nostoxanthin	
Micrococcus luteus	3	Sarcinaxanthin diglycoside	Sarcinaxanthin	
Cyclobacterium sp.				
Xanthomonas sp.				

[1] Tentative identification

[2] Co-elution of at least three carotenoids, all nostoxanthin skeletons with sugar

[3] Not selected for further analyses due to low production of the desired carotenoid

This study shows a great diversity of carotenoids in marine heterotrophic bacteria and that single strains are able to produce several different carotenoids as exemplified in Figure 3.2. Through this work, we have shown that an already established bacterial collection can have great value and is easily accessible for targeted screens like carotenoids with specific light absorption properties.

3.1.4 Selecting the carotenoid candidate

Among the isolated carotenoids, the C_{50} sarcinaxanthin was chosen as candidate for potential commercial applications, such as sun protecting agent as it was not already covered by a patent or had known issues regarding stability and toxicity that would create conflicts with a potential commercial exploitation. Examples of patented carotenoids are astaxanthin [49], zeaxanthin, β-carotene, lutein and lycopene [272]. Among several bright yellow bacterial strains in the culture collection found to produce sarcinaxanthin there was one that formed more intensively colored colonies than the others did (Figure 3.5). The isolate, designated Otnes7 was a *Micrococcus luteus* strain as revealed by 16S rRNA analysis.

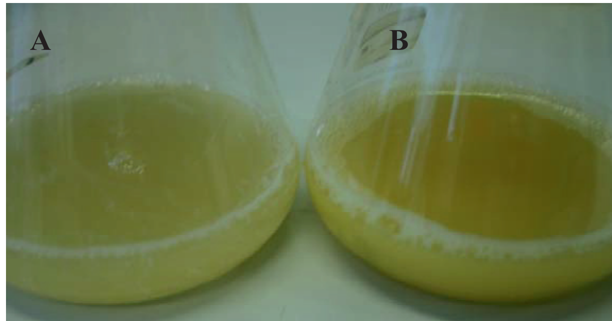

Figure 3.5
Liquid culture of two strains found to produce sarcinaxanthin.
A: Vågland1
B: Otnes7

M. luteus is a representative of the *Actinobacteria* and has a G+C content among the highest in *Bacteria* (~75%). It can be found in a variety of environments including soil, water, animals, and some dairy products. It is regarded as an interesting organism for biotechnological applications for several reasons;

- it is a catabolically versatile organism with the ability to utilize a wide range of potentially toxic substrates and therefore has great potential as a bioremediator [220, 241, 293]
- the membranes of *M. luteus* are rich in enzymes that catalyze the synthesis of prenyl pyrophosphates included long-chain alkene biosynthesis [186, 219] and might be useful in isoprene and terpene synthetic reactions as well as biofuel production [214]

In addition, it has certain characteristics that that makes it amenable for genetic engineering (to be discussed later).

3.2 Molecular cloning, expression, and functional analysis of the genes responsible for sarcinaxanthin biosynthesis in *M. luteus*

To be able to control and improve the production of sarcinaxanthin, detailed insight in the biosynthetic pathway is needed. The first step towards elucidation of the sarcinaxanthin biosynthetic pathway is to clone the presumed gene cluster in *M. luteus* strain Otnes7. Our hypothesis was that the carotenoid genes are organized in a single cluster as proven for most bacteria (see chapter 1.5.4). A genomic cosmid library representing the whole genome of *M. luteus* strain Otnes7 was constructed. The

SuperCos1 Cosmid Vector Kit and the Gigapack III XL packaging Extract (Stratagene) were used for making the cosmid library. The system is optimized to result in vectors containing 30-42 kb fragments of the partially digested chromosomal DNA. For comparison, the size of two other identified C_{50} carotenoid gene clusters are ~10 kb and ~6 kb (decaprenoxanthin gene cluster in *Corynebacterium glutamicum* and C.p 450 genecluster in *Dietzia* sp. respectively). At the start of this project, the genome size of *M.luteus* was not known. However, the size of a *M.luteus strain* had been estimated to 2.3 Mbp by the aid of restriction fragments [175]. Together with the genome sizes of other strains in the family Micrococcaceae [58], *M. luteus* strain Otnes7 was estimated to have a genome size between 2.3 and 2.5 Mbp. Implying that ~1700 colonies of the cosmid library would be sufficient for obtaining at least 20 times coverage of the genome. More than 8000 colonies were obtained. However, no pigmented clone was observed. This could signify that no clone contained the entire gene cluster for sarcinaxanthin. Considering the high coverage of the genome (more than 95 times), this was not likely. Other reasons could be that the genes for carotenoid biosynthesis were not functionally expressed or that they were not sufficiently expressed to result in visible pigmentation. With this strategy, it was not possible to identify the gene cluster for further expression of the genes in a heterologous system.

In a second approach, the work to construct a specific *crt* probe was initiated using degenerate primers based on the deduced amino acid sequences of conserved regions of CrtI proteins of other known C_{50} carotenoid producers (*Dietzia* sp., *C. glutamicum*). Several attempts of PCR amplification with various degenerate primers were made, using Block Maker and COnsensus-DEgenerate Hybrid Oligonucleotide Primers [215], but none were successful. Most likely, this was due to distant sequence similarity between the *M. luteus crt* genes and *crt* genes available in the databases.

While these attempts to find and amplify the carotenogenesis genes in *M.luteus* still were in progress, the complete genomic sequence of *Micrococcus luteus* NCTC2665 "Fleming strain" was released by the Joint Genome Institute (March 2008, www.jgi.doe.gov). This made *in silico* identification and directed PCR amplification of the *crt* genes in the Fleming strain possible. The homology of the 16S-23S rDNA internal spacer region from *M. luteus* strains is reported to be as low as 40% [86], and the heterogeneity of the whole genomic DNA level has been demonstrated by Moore

and co-workers [175]. Still, the access to the genome sequence markedly increased the chances for successful amplification of the *crt* genes also from strain Otnes7.

3.2.1 Identification of sarcinaxanthin gene cluster in *M.luteus* (Paper II)

In silico screening of the genomic sequence data of *M. luteus* strain 2665 resulted in identification of a putative carotenoid biosynthesis gene cluster consisting of totally nine open reading frames, or1007 – or1015. The genetic organization of the carotenoid (*crt*) genes in *M. luteus* displayed certain similarities to the previously published biosynthetic gene clusters for the C_{50} carotenoids C.p.450 and decaprenoxanthin in *Dietzia* sp. [260] and *C. glutamicum* [122], respectively (Figure 3.6). The deduced *M. luteus* gene products displayed between 31% and 53% primary sequence identity to enzymes of the decaprenoxanthin and C.p.450 biosynthetic pathways (Table 3.2). Although the protein identity is as high as 76% (lycopene elongase) with *C. glutamicum* and *Dietzia* sp. CQ4, no significant region of homology could be found between these organisms.

Table 3.2. *M. luteus* sarcinaxanthin biosynthetic gene cluster and primary sequence comparison with respective homologues from biosynthesis of decaprenoxanthin biosynthesis and C.p.450 in *C. glutamicum* and *Dietzia* sp. CQ4, respectively. (Table 3, Paper II)

ORF[a]	Gene name	Predicted gene product	*C. glutamicum*		*Dietzia sp. CQ4*	
			Homologue	Primary sequence indentity (%)	Homologue	Primary sequence identity (%)
or1007	*crtX*	Glycosyl transferase (CrtX)	None		CrtX	43
or1008		Unknown				
or1009	*crtYh*	C_{50} γ-cyclase subunit (CrtYh)	CrtYf	31	LbtBC[b]	38
or1010	*crtYg*	C_{50} γ-cyclase subunit (CrtYg)	CrtYe	32	LbtA	36
or1011	*crtE2*	Lycopene elongase (CrtE2)	CrtEb	50	LbtBC[c]	53
or1012	*crtI*	Phytoene desaturase (CrtI)	CrtI	43	CrtI	53
or1013	*crtB*	Phytoene synthase (CrtB)	CrtB	41	CrtB	48
or1014	*crtE2*	GGPP synthase (CrtE)	CrtE	31	CrtE	33

[a)] ORF, open reading frame.

[b)] The N-terminal region of LbtBC (amino acids 1-134) is homologous to those of *M. luteus* CrtYh and *C. glutamicum* CrtYf respectively.

[c)] the C-terminal region of LbtBC (amino acids 135-432) shows homology to *M. luteus* CrtE2 and *C. glutamicum* CrtEb, respectively.

In addition CrtE2 share high similarity to the corresponding protein sequence (prenyl transferase) in *Arthrobacter aurescens* (59% identity), CrtYg and CrtYh exhibit highest similarity to a putative C_{50} carotenoid epsilon cyclase in *Agromyces mediolanus* (47% identity) and the lycopene ε-cyclase isoprenoid transferase B, *Leifsonia xyli* (52% identity), respectively. Based on these sequence analyses the *M. luteus* genes *crtE*, *crtB*, *crtI*, *crtE2* (encoding lycopene elongase), *crtYg* (encoding C_{50} cyclase subunit), *crtYh* (encoding C_{50} cyclase subunit) and *crtX* (encoding glycosyl transferase) were assigned (Table 3.2). In addition, or1008 and or1015 encoded putative proteins with no assigned functions. To our knowledge, no analogous gene to *crtX* has been found in the *C. glutamicum* genome sequence and still this bacterium can synthesize glycosylated decaprenoxanthin [124]. The putative biosynthetic gene cluster for sarcinaxanthin is depicted in Figure 3.6 A together with the corresponding biosynthetic pathway (B).

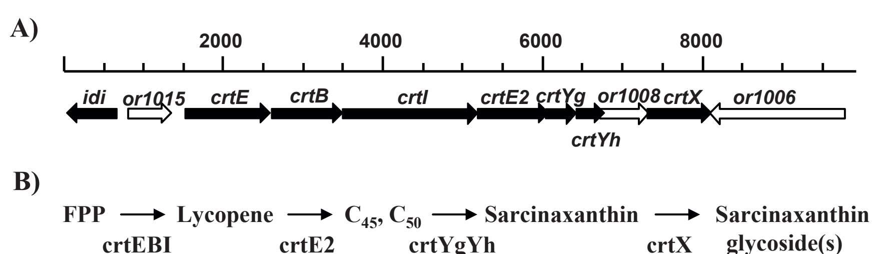

Figure 3.6 Chromosomal organization of the *M. luteus* sarcinaxanthin biosynthetic gene cluster (A). Genes indicated in white are suggested not to be involved in carotenoid biosynthesis. The biosynthetic pathway for sarcinaxanthin and its glycoside(s) as deduced from the identified gene cluster (B). (Modified from Figure 2, Paper II).

To experimentally verify that the identified *M. luteus* gene cluster encoded an active sarcinaxanthin biosynthetic pathway, the entire *crtEBIE2YgYh* region (5,8 kb fragment) from *M. luteus* NCTC2665 and Otnes7 were PCR amplified from genomic DNA using primers deduced from the NCTC2665 *crtE2* and *crtYh* gene sequences. These were first subcloned into the PGEM_T vector and after verification of correct insert cloned under transcriptional control of the positively regulated *Pm* promoter in plasmid pJBphOx [242]. The resulting plasmid were introduced into the non-carotenogenic *E. coli* host

strain XL1-blue by transformation, and the recombinant strain was analyzed for carotenoid production under induced conditions (0.5 mM m-toluic acid) over a 48 hours period. LC-MS analysis of cell extracts revealed a small peak at identical retention time, absorption spectrum, and molecular mass as sarcinaxanthin identified in the *M. luteus* strains [246]. No sarcinaxanthin was detected in plasmid free cells, thus confirming that the identified gene cluster encodes a sarcinaxanthin biosynthetic pathway starting from FFP (Figure 1.8, section 1.5.3). However, the sarcinaxanthin produced was estimated to be only 10-15 and 20-30 µg/g CDW for recombinant *E.coli* strains expressing NCTC2665 and Otnes7 derived crt genes, respectively. The corresponding values for sarcinaxanthin production it the *M. luteus* wild types were 145 µg/g CDW and 190 µg/g CDW.

3.2.2 Elucidation of the sarcinaxanthin biosynthetic pathway in *M.luteus* (Paper II)

To investigate the role of each single *crt* gene in the cluster and elucidate the biosynthetic steps from FPP to sarcinaxanthin (See Figure 3.6 B), vectors containing only modules of the crt-cluster were constructed and overexpressed in *E. coli* host strains. The DNA fragments including the genes *crtE*, *crtB* and *crtI* that together should lead to the common precursor lycopene (Figure 3.6) were amplified by PCR from both *M. luteus* strains NCTC2665 and Otnes7 and cloned in the same way as the complete gene cluster. However, lycopene production yield was low (8 - 12 µg/g CDW) and in the same range as the sarcinaxanthin production yield obtained when expressing the complete *crtEBIE2YgYh* gene cluster. This result confirmed the sequential biological functions of the three genes and suggested that these genes (*crtEBI*) might represent a bottleneck for high level production when the complete gene cluster was expressed in *E.coli*. This might be the same effect as we experienced when screening the cosmid library for pigmented clones; the expression of *M.luteus crtEBI* genes in *E.coli* were too low to result in visible color. The biological reason for the low lycopene production level remains to be further investigated. However, there are no indications from analysis of the gene sequence that the genes *crtEBI* in *M.luteus* is highly different from *crtEBI* genes from other organisms that have successfully been expressed heterologously in *E.coli*. The homologies with the corresponding genes in *C. glutamicum* and *Dietzia* sp.

CQ4 are 31-53% (Table 3.2) and the homology between *C. glutamicum* and *Dietzia* sp. CQ4 between 27 and 48% (not shown). Very recently it has been reported that *C. glutamicum* possesses two functional phytoene synthase genes (*crtB*) in two separate carotenoid gene clusters [94]. If *M.luteus* also possesses a second carotenoid gene cluster with functional genes it could explain why the heterologous expression of *crtEBI* resulted in such a low expression; wild type expression might rely on genes from the second carotenoid cluster.

Due to the low lycopene production achieved by using the *M.luteus crtEBI* genes we introduced the *crtEIB* gene cluster from *Pantoea ananatis* using plasmid pAC-LYC [167]. LC-MS analysis of the resulting strain XL1-blue (pAC-LYC) confirmed that lycopene was accumulated as sole carotenoid, and the production in this strain was ~100 times higher (1.8 mg/g CDW) than what we achieved using *M.luteus* derived genes. Therefore, it was decided not to use *M.luteus crtEBI* genes, but instead the corresponding *Pantoea ananatis* genes for further experiments.

The carotenoid biosynthesis genes *crtE2*, *crtYg* and *crtYh* putatively encode for the proteins that catalyze the formation of sarcinaxanthin from the precursor lycopene (Figure 3.6). To experimentally verify their function, these genes from both *M. luteus* strain (Otnes7 and NCTC2665) were cloned into pJBphOx (pCRT_E2YgYh) and introduced into the lycopene producing XL1-blue (pAC-LYC) for color complementation experiments and analysis of sarcinaxanthin biosynthesis. LC-MS analysis of the resulting yellow cell extracts revealed a total maximum carotenoid accumulation of 2.3 mg/g CDW and about 98% of the total carotenoid produced was identified as sarcinaxanthin in recombinant strain expressing NCTC2665 genes and 2.5 mg/g CDW and 100% sarcinaxanthin in the strain expressing Otnes7 genes. These data demonstrated that the *M. luteus crtE2YgYh* gene products can effectively convert lycopene into sarcinaxanthin.

To identify the intermediate metabolites between lycopene and sarcinaxanthin, two vectors were constructed; one contains the gene coding for lycopene elongase (*crtE2*, Figure 3.6), potentially producing C_{45} and C_{50} precursor carotenoids and the second containing in addition the first gene encoding for C_{50} cyclase subunit (*crtE2Yg*). Genes from both *M. luteus* strains were used. These were then expressed in *E. coli* with lycopene producing background. In *C.glutamicum* and *Dietzia* sp. CQ4 C_{50}

biosynthesis, both C_{50} cyclase subunits were required to form an active catalytic unit [122, 260]. Therefore, it was not surprising that these two gene products (CrtE2 and CrtE2Yg) resulted in the formation of the same carotenoid profile and no sarcinaxanthin, as revealed by HPLC analysis. Two different carotenoids accumulated in these cells in addition to lycopene (Figure 3.7 D); all three compounds sharing identical UV/Vis profiles. The minor carotenoid had molecular mass of 620 Da, indicating a C_{45} xanthophyll compared with reported values (620-624 Da), and the major carotenoid had a molecular mass of 704 Da indicating a C_{50} xanthophyll (704-738 Da). Only a small number of C_{50} and C_{45} carotenoids are known and a tentative identification of the carotenoids as flavuxanthin (peak 5, Figure 3.7 D) and nonaflavuxanthin (peak 6) based on their DAD profile and molecular mass was made. NMR confirmed the identity of the major carotenoid as acyclic C_{50} carotenoid flavuxanthin [124].

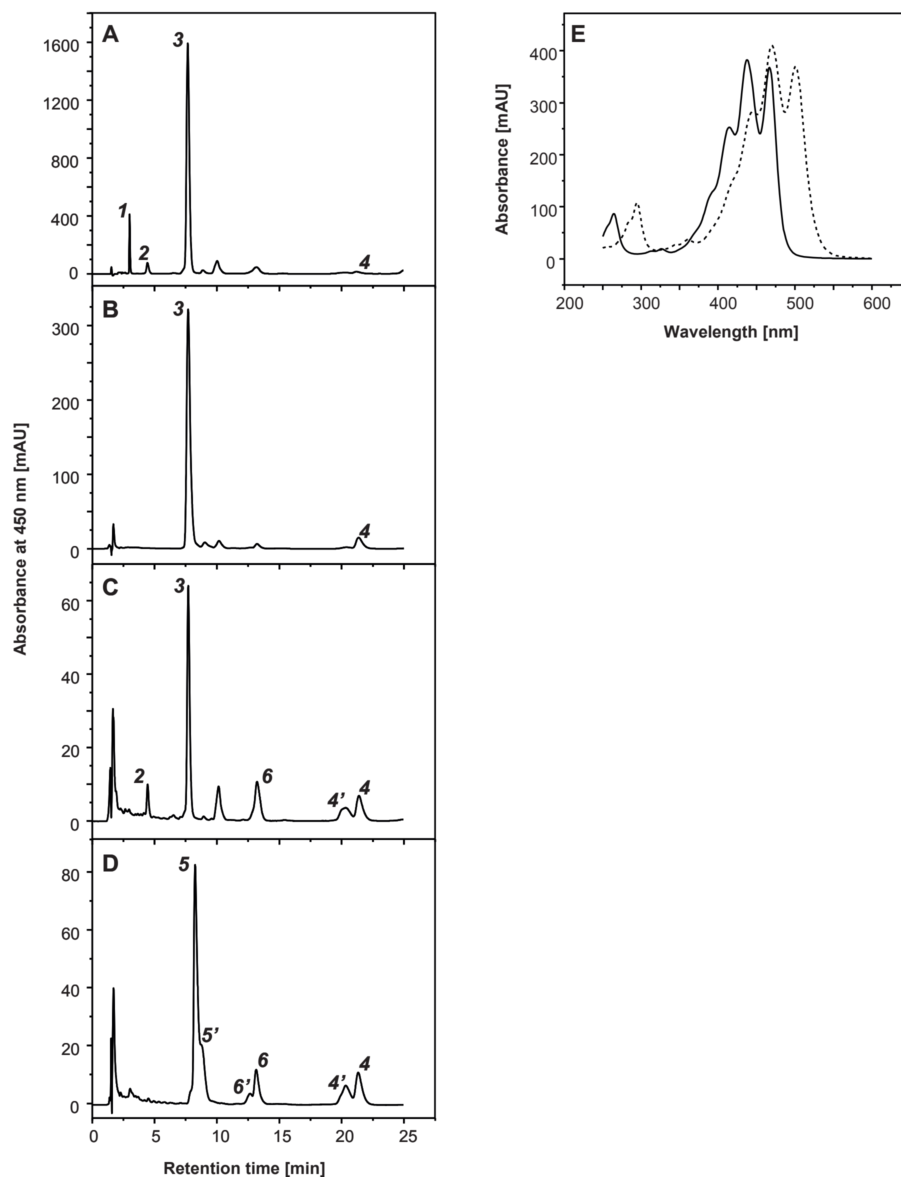

Figure 3.7. HPLC elution profile of carotenoids extracted from *M. luteus* strain Otnes7 (A), lycopene-producing *E. coli* XL1 Blue (pAC-LYC) transformed with pCRT-E2YgYh-O7 (B), pCRT-E2YgYhX-O7 (C) and pCRT-E2-O7 (D). Peak 1, sarcinaxanthin diglucoside; peak 2, sarcinaxanthin monoglucoside; peak 3, sarcinaxanthin; peak 4, lycopene; peak 5, flavuxanthin; peak 6, nonaflavuxanthin; Peak 4' 5' and 6' are the *cis* isomers of 4, 5 and 6 respectively. Absorption spectra of carotenoids from peaks 1, 2 and 3 (solid line) and peaks 4, 5 and 6 (scattered line) are depicted in graph (E). (Figure 1, Paper II).

These results verified that the *M. luteus crtE2* gene encodes a lycopene elongase catalyzing the sequential elongation of the C_{40} carotenoid lycopene via the C_{45} carotenoid nonaflavuxanthin to the C_{50} carotenoid flavuxanthin. In light of these results, we could also conclude that the cyclase subunit crtYg is not active alone but must act together with CrtYh as an active carotenoid cyclase catalyzing cyclization of flavuxanthin to sarcinaxanthin *in vivo*. To analyse the specificity of this carotenoid cyclase, lycopene producing *E.coli* strain were transformed with pCRT-YgYh expressing the genes encoding for the carotenoid cyclase subunits (*crtYgYh*) from both *M. luteus* strains. HPLC analysis of cell extracts showed that these strains accumulated lycopene as the only carotenoid (data not shown), confirming that the *crtYgYh* gene products can not use lycopene as a substrate *in vivo*. Together, these data confirmed that CrtYg and CrtYh polypeptides together constitute an active γ-type C_{50} carotenoid cyclase catalyzing cyclization of flavuxanthin to sarcinaxanthin *in vivo.*

Finally, the function of *crtX* was to be experimentally verified. The *crtX* gene product would be the first functionally verified glycosyl transferase involved in carotenoid biosynthesis and the *crt* gene cluster would be expanded. We constructed strain XL1 Blue (PAC-LYC) (pCRT-E2YgYhX-O7) with genes from *M. luteus* strain Otnes7. This strain would express *crtX* in addition to the genes leading to sarcinaxthin. HPLC analysis of the cell extract (Figure 3.7 C) revealed sarcinaxanthin as the major carotenoid (peak 3), but in additional a more polar carotenoid was eluted earlier (peak 2) which had identical retention time and absorption spectrum to that of sarcinaxanthin monoglucoside from *M. luteus* Otnes 7 (results not shown). Another minor peak was observed with the same retention time as that of sarcinaxanthin diglycoside produced by *M. luteus* strains [246]; however, the detected amount was too low for a confident analysis of the mass and absorption spectrum. About 10% of the produced sarcinaxanthin was glucosylated both in *M. luteus* wild-type strains and when produced heterologous in *E. coli*, under the conditions tested. These results confirmed that *crtX* encodes an active glycosyl transferase that is necessary for the glycosylation of sarcinaxanthin and the complete biosynthetic pathway of sarcinaxanthin and its glucosides from FFP and via lycopene in *M. luteus* could be presented as in Figure 3.8.

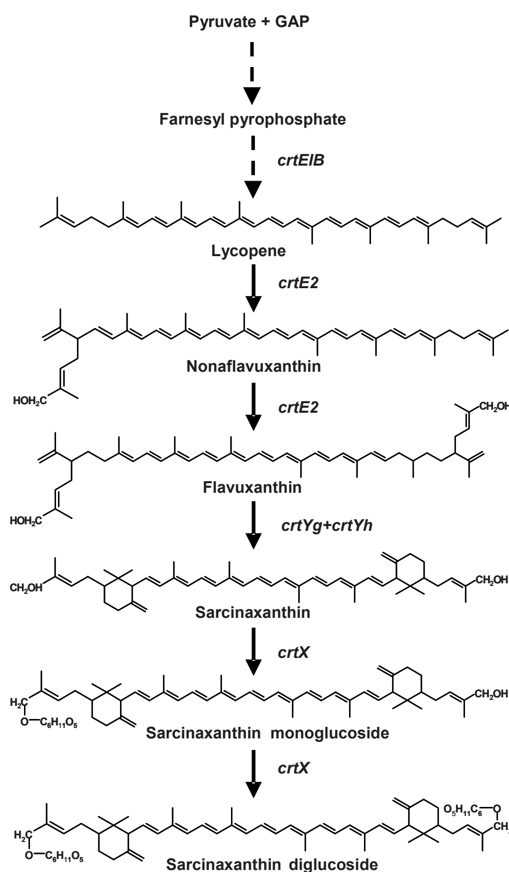

Figure 3.8. The biosynthetic pathway for the individual steps in the formation of sarcinaxanthin and its glycosides from lycopene. CrtEBI, GGPP synthase, phytoene synthase, and phytoene desaturase; CrtE2, lycopene elongase; CrtYg plus CrtYh, C_{50} carotenoid γ–cyclase; CrtX, C_{50} carotenoid glycosyl transferase (Figure 4, Paper II).

3.2.3 Comparison of sarcinaxanthin genes from strain Otnes7 and 2665 (Paper II)

The Otnes7 strain was chosen among several other marine *M.luteus* strains due to the more intense color in liquid culture (Figure 3.4). Otnes7 was also a more efficient sarcinaxanthin producer than the Fleming strain, confirmed both by visual observation and by measuring the accumulated carotenoid content, with 24% higher production in the Otnes7 strain. However, when produced heterologously, the difference was only 8.7%. This indicates that lycopene synthesis is a bottleneck in improving heterologous

sarcinaxanthin production with Otnes7 derived genes under conditions tested. This difference between the strains was more pronounced when only the lycopene elongase gene (*crtE2*) was overexpressed in *E.coli* in a lycopene producing background. Seventy-nine % of the total carotenoid was flavuxanthin when overexpressing the *crtE2* gene deriving from Otnes7 and only 23 % when overexpressing the *crtE2* gene deriving from the NCTC2665 strain. Remaining fractions were lycopene and the intermediate nonaflavuxantin. Therefore, to further compare the efficiency of Otnes7 versus NCTC2665 derived biosynthetic genes, carotenoid production analyses were performed with different *Pm* inducer concentrations. The results demonstrated that strain XL1-blue (pAC-LYC) (pCRT-E2YgYh-O7) produced sarcinaxanthin to significantly higher levels than XL1-blue (pAC-LYC) (pCRT-E2YgYh-2665) under all conditions tested, thus confirming that Otnes7 genes are preferable for efficient sarcinaxanthin production in *E. coli* hosts (Figure 3.9). DNA sequence analysis of the cloned Otnes7 *crtE2YgYh* fragment revealed totally 24 nucleotide substitutions compared to corresponding NTCT2665 DNA sequence, corresponding to four amino acid substitutions in CrtE2, five in CrtYg, and two substitutions plus one insertion in CrtYh. Whether these sequence variations positively affects the expression level or the catalytic properties of the respective proteins remained unknown, and this was no further investigated.

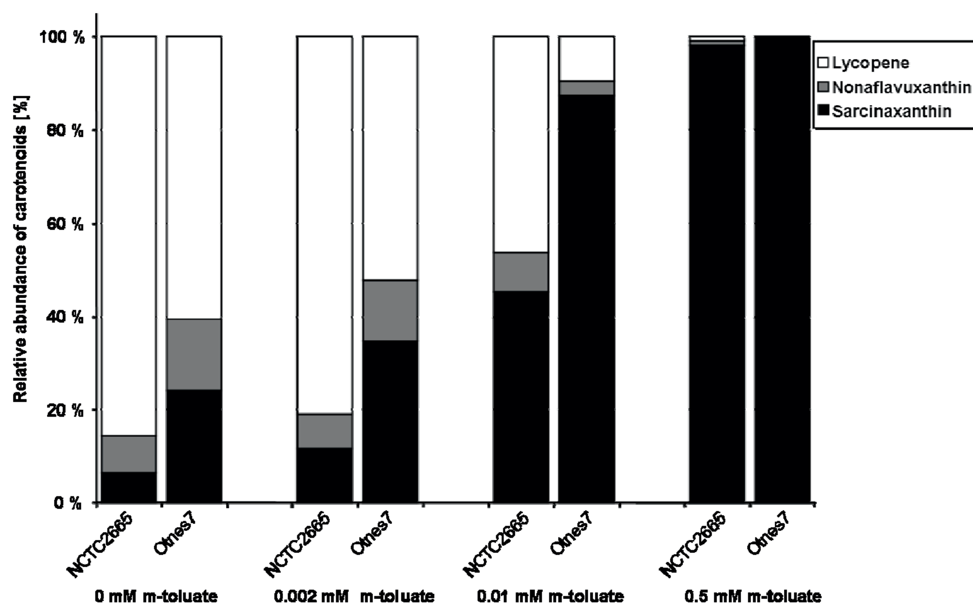

Figure 3.9. Relative carotenoid abundances in extracts from E. coli(pAC-LYC)(pCRT-E2YgYh-O7) and E. coli(pAC-LYC)(pCRT-E2YgYh-2665) overexpressing crtE2, crtYg, and crtYh genes from M. luteus strains Otnes7 and NCTC2665 cultivated in the presence of various Pm inducer concentrations (0, 0.002, 0.01, and 0.5 mM m-toluic acid). The fractions of sarcinaxanthin, lycopene, and intermediates are indicated. (Figure 3, Paper II).

3.2.4 A novel carotenoid, sarprenoxanthin, was synthesized using combinatorial biosynthesis (Paper II)

The biosynthetic pathway for decaprenoxanthin in *C. glutamicum* is reported to involve cyclization of flavuxanthin by an ε-cyclic C_{50} cyclase encoded by *crtYe* and *crtYf*. This means that the biochemical functions of the C_{50} cyclase proteins in *M. luteus* and *C. glutamicum* are the major differences between the pathways. Flavuxanthin was proven the branch point in the diversification of sarcinaxanthin and decaprenoxanthin. To experimentally verify this, we established and analysed *E. coli* XL1 Blue (pAC-LYC) (pCRT-E2-O7-YeYf-MJ) expressing a hybrid operon containing *crtE2* from Otnes7 and *crtYe* and *crtYf* from *C. glutamicum* MJ233-MV10 (Figure 3.10).

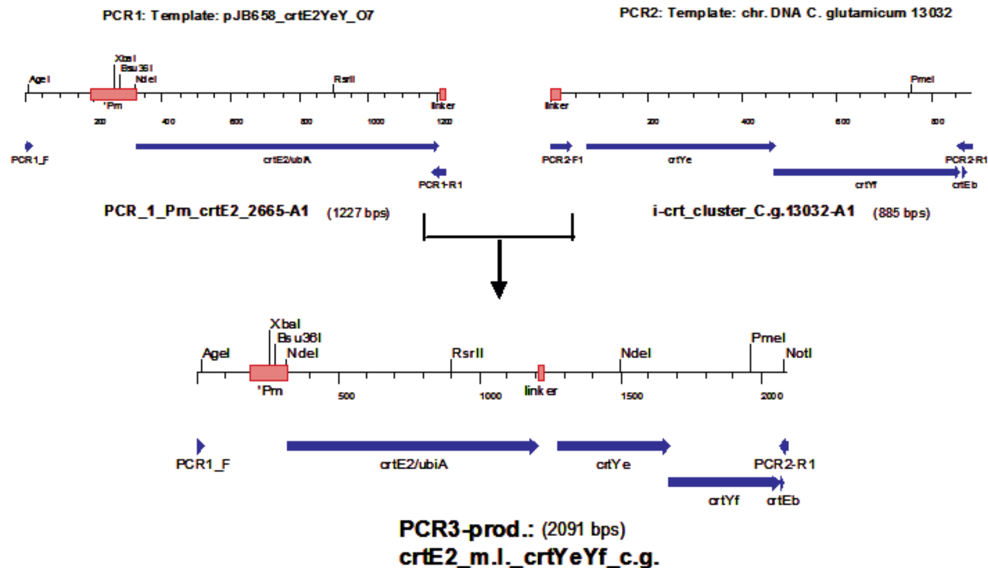

Figure 3.10. In vitro design of a hybrid operon containing *crtE2* from *M.luteus* and *crtYeYf* from *C.glutamicum*.

We expected this recombinant strain to produce decaprenoxanthin. Surprisingly, LC-MS analysis revealed that three different cyclic C_{50} carotenoids were accumulated, exhibiting the same UV/Vis absorbance spectre and mass (Figure 3.11). The retention time for peak 1 was identical to that of sarcinaxanthin. Peaks 1, 2 and 3 represented 35%, 46%, and 19%, respectively, of the total carotenoid content in the recombinant cells. No lycopene was detected.

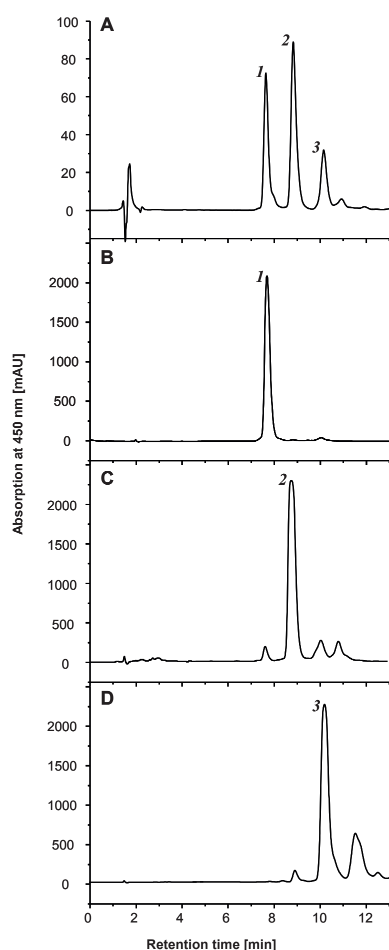

Figure 3.11. HPLC elution profiles of the carotenoids extracted from *E. coli* (pAC-LYC)(pCRT-E2 ml-YeYfcg) (A); purified peak 1, sarcinaxanthin (B); peak 2, sarprenoxanthin (C); and peak 3, decaprenoxanthin (D). (Figure 5, paper II).

All three fractions were then purified and analyzed by NMR. The NMR data confirmed the identity of peak 1 as sarcinaxanthin [132], while peak 3 was identified as decaprenoxanthin [77, 79]. The NMR data of the purified peak 2, representing the major product, indicated a molecule that cannot be clearly distinguished from a 1:1 mixture of sarcinaxanthin and decaprenoxanthin, so its existence had to be confirmed by chromatographic methods. The purified fractions were reanalyzed by LC using a high resolution method to control its purity and the corresponding chromatogram clearly shows that fraction 2 is a distinct carotenoid different from fraction 1 and 3 (Figure 3.11 B and C). Peak 2 was identified as a new bicyclic asymmetric C_{50} carotenoid with the systematic name 2,2′-Bis(4-hydroxy-3-methyl-2-butenyl)-γ, ε–carotene. The compound

exhibits one ε-cyclic and one γ-cyclic structure and thus appears to be a structural combination of sarcinaxanthin and decaprenoxanthin. This is in total agreement with the polarities of the three carotenoids (Figure 3.11), and the compound was named sarprenoxanthin (Figure 3.12). To rule out that these results were not due to any unforeseen functions of the hybrid operon as such, we established analogous strain *E. coli* XL1 Blue (pAC-LYC) (pCRT-YeYfEb-MJ) expressing decaprenoxanthin genes *crtYe, crtYf and crtEb* from *C. glutamicum* (see Figure 3.5). The resulting chromatographic profile and absorbance spectres were the same as in the hybrid construct with identical relative abundance, mass, UV/Vis profile and retention time of the carotenoids (data not shown). Moreover, DNA sequencing confirmed that the expressed *crt* genes were wild-type *C. glutamicum* sequences as described in the literature (41). Together, these results revealed that the *crtYe* and *crtYf* genes encoded a multifunctional C_{50} carotenoid cyclase that can catalyze synthesis of three different bicyclic carotenoids. To our knowledge, this is the first time reported that *C. glutamicum* genes can be used to synthesize sarcinaxanthin and decaprenoxanthin.

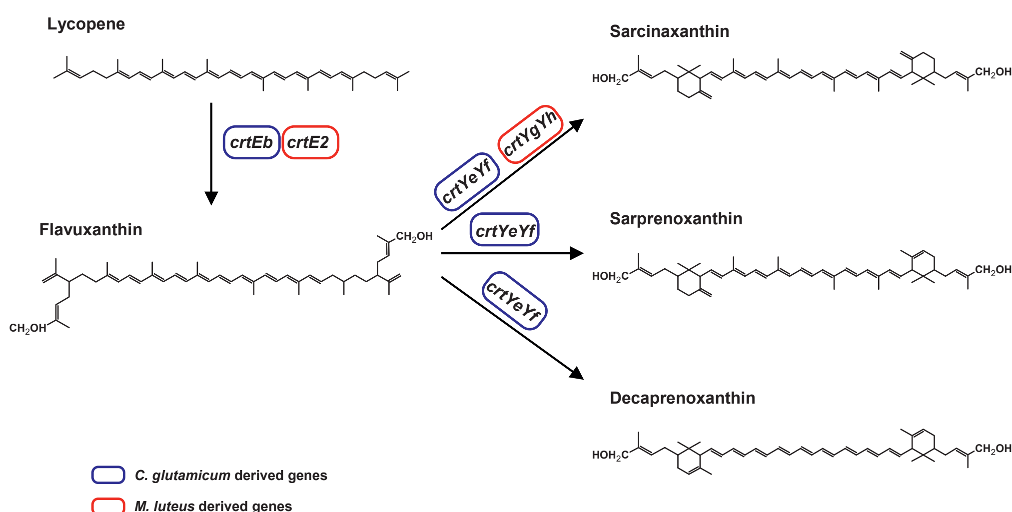

Figure 3.12. Diverse biochemical functions of the *M. luteus* and the *C. glutamicum* C_{50} carotenoid cyclases. Both sarcinaxanthin and decaprenoxanthin biosynthesis involve conversion of lycopene to flavuxanthin catalyzed by lycopene elongases CrtEb and CrtE2 in *M. luteus* and *C. glutamicum*, respectively. The *M. luteus* CrtYgYh polypeptides constitute a γ-cyclase specifically converting flavuxanthin into sarcinaxanthin. In contrast, the *C. glutamicum* CrtYgYh polypeptides constitute both γ-cyclase and ε-cyclase activity and can convert flavuxanthin into three different C_{50} carotenoids; decaprenoxanthin, sarcinaxanthin, and sarprenoxanthin. (Figure 6, Paper II)

3.3 Increased sarcinaxanthin production

For industrial scale heterologous production processes to be established, the first topics to solve are increasing the carotenoid titers and establishing a stable fermentation process without selective pressure. When using *E.coli* as host for heterologous gene expression under the control of the adjustable Pm promoter in small scale laboratory conditions up to 10-fold higher sarcinaxanthin production levels was achieved compared to the levels obtained by *M. luteus* strains. However, there is great potential to increase the carotenoid production further. Evident targets for increasing the precursor supply for lycopene production were identified from the literature (section 1.5.6, Figure 1.7). These being overexpression of *dxs* and *dxr* in the DOXP pathway, the introduction of the MVA pathway and overexpression of *idi* and FPP synthase, common for both pathways (Figure 1.8), in addition to the *crt* genes *crtE* and *crtB*.

3.3.1 Introduction of the MVA pathway (unpublished results)

E. coli strains carrying an exogenous MVA+ operon were reported to have a higher lycopene production upon induction (arabinose) when mevalonate was present in the media [212]. These strains, EcAB4-1(K12 and EcAM5-1(BL21), were kindly supplied by the authors and transformed with pLYC and pCRT_E2YgYh-O7 or with pLYC and pCRT_E2YgYh-2665. All strains were induced with the standard concentration 0,5mM m-Toluate, and 0,1 % arabinose.

In strain EcAB4-1 we obtained a 2-fold increase in the total carotenoid production but surprisingly no increased sarcinaxanthin production. In strain EcAM5-1, the total carotenoid production was less than in XL1-Blue background. For comparison, the same strains containing only plasmid pAC-LYC were tested. In strain EcAB4-1, the total carotenoid production was the same as when containing the pCRT vector, but for strain EcAM5-1 a three times higher total carotenoid production was measured when only plasmid pAC-LYC was present. Strain EcAM5-1 was therefore excluded from further experiments. The effect of higher m-toluate concentrations was tested on EcAB4-1 and XL1-Blue strains to test whether the remaining lycopene could be transformed to sarcinaxanthin upon higher induction. However, as expected, the presence of m-toluate concentrations higher than 1 mM negatively affected the growth of EcAB4-1.

These results suggest that the tested new lycopene overproducing strains are not compatible with our inducer system for the pJBphOx vector. In addition, the reported values for lycopene production were not achieved in these strains containing only the pLYC plasmid. These results in combination with the cost of mevalonate (~8000 NOK per 10 mg) made also these strains not interesting for further experiments.

3.3.2 The pJBphOx vector system containing *crt* genes is stably maintained in the cells (unpublished results)

To be able to produce carotenoids on a larger scale it will be necessary to cultivate selected strains in fermentors under high cell density conditions. A presumption for this type of cultivation is the absence of antibiotics. Therefore, high plasmid stability under maximal permissive conditions is necessary. It has earlier been demonstrated that the

pJBphOx vector system used for heterologous gene expression in *E.coli* host strains is stably maintained in the cells during lab bench scale high-cell density fermentation, but this may change depending on the protein expressed [242].

Initial tests of the plasmid stability in XL1 (pAC-LYC) (pCRT_E2YgYh-O7) and EcAB4-1 (pAC-LYC) (pCRT_E2YgYh-O7) were performed. The strains were tested for plasmid stability in shake flask cultivations without antibiotics added. After 13 generations, still 98% of the cells contained both plasmids and formed yellow colored colonies on LB agar plates. The majority on the remaining 2% were colorless (lack either plasmids or only pAC-LYC) and a small fraction were red/pink (lack pCRT_E2YgYh-O7). This is a strong indication that this vector system for sarcinaxanthin production can be used for high cell density fermentations under permissive conditions.

3.3.3 Potential of utilizing *M. luteus* as the producing organism (unpublished results)

M.luteus is regarded an interesting organism for biotechnological applications for several reasons, like it's potential use in bioremediation and the high amount of enzymes in the membrane that catalyzes the synthesis of prenyl pyrophosphates (Section 3.1.4). Even though these enzymes have been proposed as useful in isoprene synthetic reactions, the potential of *M. luteus* strains for industrial carotenoid production has not been explored so far. To manipulate the sarcinaxanthin biosynthesis and direct the carbon flux towards carotenoid and sarcinaxanthin production, development of genetic tools, for instance vectors for selection and overexpression, is indispensable. With the entire genomic sequence available, metabolic network modelling can be very useful. A network model was recently presented as a tool that can be used for design of an engineered *M. luteus* strain with improved alkane production [214]. The same approach can be used for designing a *M. luteus* strain with improved carotenoid production.

An important requirement for genetic work with bacteria is a method to introduce and functionally express DNA, such as introduction of vectors into a host strain. An efficient and convenient method is electroporation of competent receptor

cells. However, the construction of vector systems for selection and overexpression is indispensable. A shuttle vector replicates in two different organisms and the advantage is that it can easily be amplified and genetically manipulated in *E.coli* and thereafter transferred back to *M.luteus* for recombinant gene expression (e.g. enhanced carotenoid production).

Due to the phylogenetic relationship between the genera *Micrococcus* and *Corynebacterium* attempts were made to generate electrocompetent cells of *M. luteus* strain Otnes7 according to a modified method for *C. glutamicum* [114]. The potentially electrocompetent cells were then attempted to be transformed according to a *C. glutamicum* method as well with several shuttle vectors found to replicate in both *E.coli* and Gram-positive bacteria [48, 93, 104, 137, 174, 195]. After initial attempts, no transformations were successful, and in order to succeed a lot of fine-tuning of the protocols would have been necessary. Without the certainty that the shuttle vectors selected were able to replicate in *M. luteus*, it was decided not to continue with this work.

M.luteus Otnes7 plasmid as a potential backbone for developing of genetic tools for _M. luteus_

When analyzing chromosomal DNA from *M.luteus* Otnes7 with gel electrophoreses a significant band appeared on the gel (Figure 3.13), and the hypothesis was that this band represented a natural plasmid.

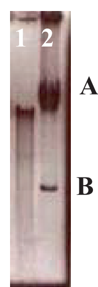

Figure 3.13. Gel electrophoresis of isolated chromosomal DNA from *M.luteus* digested with *Sau3a*I. Undigested chromosomal DNA (A) and putative plasmid DNA (B). Lane 1: partially digested DNA, lane 2: undigested DNA.

To isolate the plasmid DNA the Genopure plasmid midi kit from Roche was used with some modifications. A 100 ml overnight culture was resuspended in the suspension buffer/RNAse solution following the kit and the lysis buffer was replaced with adding 80 µl mutanolysin (2000U/ml), 80 mg lysozyme and 4 g sucrose (2,5%) [111]. The yield was 18 µg plasmid from 100 ml culture. The potential plasmid DNA was analyzed using various restriction enzymes and cutting with *Pst*1 resulted in one linear fragment that was sequenced. The results from the DNA sequencing (primer walking) was a ~3.6 kbp fragment (which represents a complete linear version of this plasmid) and is depicted in Figure 3.14.

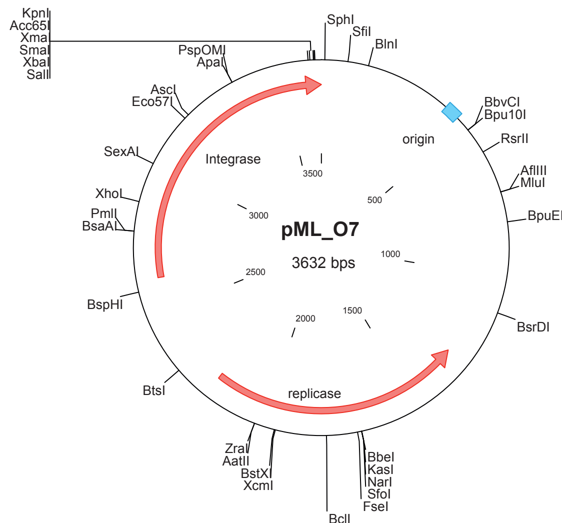

Figure 3.14 Map of the *M. luteus* Otnes7 natural plasmid pML_O7. Putative origin for replication (blue), putative integrase(red) and putative replicase (red) are indicated.

A putative origin for replication as well as a replication initiation protein gene was identified; these two genetic elements may be highly useful to construct an *E. coli-M. luteus* shuttle vector for future gene delivery in *M. luteus*. In addition, a putative gene encoding an integrase/transposase was identified. The small size of this plasmid makes it extra interesting, as it will minimize the metabolic burden by replication.

Twenty percent of all *Micrococcus luteus* strain are reported to contain plasmid(s), in the size range 1 to 20 MDa [160] and antibiotic resistant phenotypes are often linked to these plasmids [147, 263]. The pMEC2 plasmid, conferring erythromycin resistance and purified from a *M.luteus* strain have been successfully inserted to plasmid-free strains of *M.luteus* and *C.glutamicum* resulting in erythromycin resistance [147].

Further work on the plasmid was not initiated. However, future work would be to construction of a *M.luteus* shuttle vector starting from pML_O7 and/or other pML vectors reported in the literature and thereafter fuse it with an *E.coli* vector including selection markers. In addition, the specificity of glycosyl transferase and the effect of overexpression of *crtX* from *M.luteus* remain to be explored.

3.4 MALDI-TOF MS based taxa identification and dereplication tool for efficient screening for novel pigmentation

In the first part of this PhD project a substantial fraction of the marine heterotrophic bacterial collection was analyzed for pigments by using LC-DAD-MS analysis (Paper I). We experienced a heavy workload resulting in many redundant pigment profiles. Identical pigment profiles were suspected to be produced by the same species as also morphological characteristics (shape, consistency, motility) were the same, but no taxonomic classification was performed except the 16S rRNA analysis of six selected strains.

Of the initial collection of more than 10 000 heterotrophic bacteria, ~20% were pigmented, meaning that the largest fraction was the unexplored unpigmented bacteria. For better characterizing the bacterial collection it was of interest to look into the diversity of bacteria belonging to these two groups – pigmented vs. non-pigmented by using MALDI-TOF MS bacterial fingerprinting and in the same way verify if the pigment profiles as revealed by LC-DAD-MS could be connected to bacterial taxonomy. In addition, some pigments are produced only under certain conditions (like prodigiosin) and a screen on only pigmented bacteria would therefore not be sufficient to discover all pigment producing strains.

3.4.1 MALDI-TOF MS analysis for species differentiation (Paper III)

A hierarchically clustered dendrogram was created based on MALDI-TOF MS spectra from 373 bacterial isolates (as shown in Figure 2, Paper III). Fifty-five different clusters were identified when using the "default" distance level (Table 3.3). Twenty-one clusters contained only one isolate. In addition, 15 clusters were classified as low abundant taxa (less than 1% of the total population but above 1 isolate, i.e. two to four isolates). This confirms a large diversity of bacterial isolates in the culture collection.

Table 3.3 Overview of MALDI-TOF MS analysis divided in pigmented and non-pigmented groups. (Modified from Table 1, Paper III)

	Total	Pigmented (%)	Non-pigm. (%)	Mixed pigm. (%)
Bacterial isolates	373	243 (65)	130 (35)	
Total clusters	55	45 (82)	9 (16)	1(2)
Cluster with 2-4 isolates	15	13 (87)	2 (13)	
Cluster with 1 isolate	21	19 (91)	2 (9)	

Sixty-five percent of the 373 analysed isolates were pigmented and distributed among 45 clusters (i.e. representing 82 % of the clusters). Furthermore, as the non-pigmented isolates constituted 35% of the MALDI-TOF analysed samples were distributed in only 9 clusters (16%) and the percentage further diminishing when comparing low abundant clusters (13%) and clusters with one isolate (9%) it shows that the selection of pigmented bacteria have a greater diversity than non-pigmented bacteria. Only one cluster comprised both pigmented and non-pigmented isolates. Eight clusters were identified at the species level. The low identification percentage was as expected as most isolated in the Biotyper database is validated for clinical isolates [65, 281].

3.4.2 Pigment profile clustering shows good correlation with MALDI-TOF MS clustering (Paper III)

The MALDI-TOF MS generated dendrogram was used as aid in selecting isolates for pigment analysis. Both single bacterium clusters and multiple bacteria clusters were represented and in total 97 pigmented isolates were selected for pigment analysis by LC-DAD-MS. Eighty-two unique pigments (i.e. unique absorption spectrum, m/z and retention time) were detected among the 97 analysed isolates. The number of pigments per isolate varied from 1 to 15, and most isolates produced between 3 and 5 pigments.

This clearly shows the large pigment diversity among the various isolates. Identified pigments were zeaxanthin, nostoxanthin, lycopene and prodigiosin (based on authentic samples, earlier identification (Paper I) or strain identification (*Serratia plymuthica*)).

A matrix of pigments vs. isolates amenable for statistical analysis was constructed based on the LC-DAD-MS analysis. Thereafter a pigment dendrogram was created with the Unscrambler software (Average linking clustering). The resultant dendrogram comprised 28 pigment profiles and connections to the corresponding MALDI-TOF MS bacterial dendrogram containing 31 clusters were manually performed (as shown in Figure 4, Paper III. Eight of the pigment profiles, comprising 35 isolates, have total correlation with bacterial clusters. In addition, five single membered clusters were detected. Furthermore, five pigment profiles can be found in more than one bacterial cluster. This was as expected for pigment profiles containing less than three pigments as most marine pigments are not unique for one bacterial species and the production of one or two pigments gives a weak basis for a bacterial fingerprint. This is also consistent with research from secondary metabolites in filamentous fungi where individual metabolites have been found in both phylogenetically closely related and distantly related species [133]. In all the above mentioned clusters, no unique pigment profile would be lost if MALDI-TOF MS dereplication was applied to reduce the number of isolates for further pigment screening by LC-MS, these represent 90 % of the isolates. There is only one bacterial cluster with several pigment profiles (three). In this case only, the MALDI-TOF MS would fail as a dereplication tool, as one would risk losing unique pigments by selecting just a few isolates for further LC-DAD-MS screening. In view of the massive advantage with introducing a dereplication step before pigment analysis, this potential 10% loss in diversity would be acceptable.

4 Concluding remarks

The sea surface microlayer in the Trondheim fjord is rich in pigmented heterotrophic bacteria producing a great diversity of carotenoids as shown by the high throughput LC-DAD-MS protocol developed. The establishment of this culture collection is a valuable source for pigments with potential novel properties from unidentified bacterial species. Further, the high throughput LC-DAD-MS method can be used for efficient screening of bacterial extracts for pigments with specific UV/Vis characteristics.

A bright yellow strain, Otnes7, was identified as the sarcinaxanthin producing *M. luteus*. The biosynthetic pathway for sarcinaxanthin synthesis was elucidated, and starting from the precursor farnesyl pyrophosphate (FPP), identified intermediates were lycopene, nonaflavuxanthin and flavuxanthin. In addition, a glycosyl transferase (CrtX) was identified and functionally expressed, catalyzing the conversion of sarcinaxanthin to sarcinaxanthin mono- and diglycosid. The *M.luteus* strain Otnes7 was shown to have more efficient lycopene transforming genes than the compared *M. luteus* strain NTCT2665. The wild type production of sarcinaxanthin was 24% higher in strain Otnes7 and heterologous expression of the gene encoding for lycopene elongase (crtE2) resulted in more pronounced difference between the two strains. This suggested that CrtE2 is the main contributor to this difference and Otnes7 based genes should be chosen when aiming for a more efficient sarcinaxanthin production.

With flavuxanthin being the common substrate for the C_{50}–cyclases in *M.luteus* and *C. glutamicum* (synthesizing sarcinaxanthin and decaprenoxanthin respectively), we combined the lycopene elongase gene (*crtE2*) from *M. luteus* and the C_{50}-cyclase genes from *C.glutamicum* (*crtYe* and *crtYf*) to form a hybrid operon to further experimentally prove the difference in the last step of the biosynthesis. This combination lead to the formation of three different bicyclic C_{50} products; sarcinaxanthin, decaprenoxanthin and the new carotenoid sarprenoxanthin. These results proved that *crtYe* and *crtYf* encoded a multifunctional C_{50} carotenoid cyclase and contributed to new insight into the multiple functions of bacterial C_{50} carotenoid cyclases as key catalysts for the synthesis of structurally different carotenoids.

Production of carotenoids from marine heterotrophic bacteria is usually not industrial interesting due to low production levels. There are several strategies for optimizing the production of carotenoid pigments. Optimal cultivating conditions with

respect to carotenoid production can result in significant improvements, but the main potential lies within deregulation of metabolic pathways in order to increase the metabolic flux to isoprenoid precursors. Carotenoid storage in the heterologous host *E.coli* is a potential major bottleneck for increased carotenoid production and therefore it is important to exploit carotenoid production also in the native host by developing genetic tools like shuttle vectors. Microbial carotenoid production is a field of strong academic and industrial interests and the isolation and characterization of new carotenogenic genes expands the possibilities for heterologous production of a broad range of carotenoids.

By analyzing part of the cultural collection five years after the original screen for pigments, the viability and pigmentation was still high and the collection could be further explored. When new efficient screening strategies and analytical tool are being developed, already established bacterial collections can get added value important for successful bioprospecting. MALDI-TOF-MS analysis showed that the bacterial collection is a diverse collection of marine heterotrophic bacteria, both pigmented and non-pigmented and that pigment profiling has potential to be used as taxonomic marker at the species level when the isolates produce at least three different pigments. In addition, we showed that by using MALDI-TOF MS as dereplication tool before pigment profiling of the isolates massive redundant analysis could be avoided without compromising the diversity of the collection.

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Paper I

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Paper II

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Biosynthetic Pathway for γ-Cyclic Sarcinaxanthin in *Micrococcus luteus*: Heterologous Expression and Evidence for Diverse and Multiple Catalytic Functions of C_{50} Carotenoid Cyclases▽†

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We report the cloning and characterization of the biosynthetic gene cluster (*crtE*, *crtB*, *crtI*, *crtE2*, *crtYg*, *crtYh*, and *crtX*) of the γ-cyclic C_{50} carotenoid sarcinaxanthin in *Micrococcus luteus* NCTC2665. Expression of the complete and partial gene cluster in *Escherichia coli* hosts revealed that sarcinaxanthin biosynthesis from the precursor molecule farnesyl pyrophosphate (FPP) proceeds via C_{40} lycopene, C_{45} nonaflavuxanthin, C_{50} flavuxanthin, and C_{50} sarcinaxanthin. Glucosylation of sarcinaxanthin was accomplished by the *crtX* gene product. This is the first report describing the biosynthetic pathway of a γ-cyclic C_{50} carotenoid. Expression of the corresponding genes from the marine *M. luteus* isolate Otnes7 in a lycopene-producing *E. coli* host resulted in the production of up to 2.5 mg/g cell dry weight sarcinaxanthin in shake flasks. In an attempt to experimentally understand the specific difference between the biosynthetic pathways of sarcinaxanthin and the structurally related ε-cyclic decaprenoxanthin, we constructed a hybrid gene cluster with the γ-cyclic C_{50} carotenoid cyclase genes *crtYg* and *crtYh* from *M. luteus* replaced with the analogous ε-cyclic C_{50} carotenoid cyclase genes *crtYe* and *crtYf* from the natural decaprenoxanthin producer *Corynebacterium glutamicum*. Surprisingly, expression of this hybrid gene cluster in an *E. coli* host resulted in accumulation of not only decaprenoxanthin, but also sarcinaxanthin and the asymmetric ε- and γ-cyclic C_{50} carotenoid sarprenoxanthin, described for the first time in this work. Together, these data contributed to new insight into the diverse and multiple functions of bacterial C_{50} carotenoid cyclases as key catalysts for the synthesis of structurally different carotenoids.

Carotenoids are natural pigments synthesized by bacteria, fungi, algae, and plants, and more than 750 different carotenoids have been isolated from natural sources (17). They possess important biological functions as protectants against light and oxygen excess in photosynthetic processes (32, 38), and they have been proposed to reduce the risk of certain cancers, cardiovascular disease, and Alzheimer disease due to their antioxidative properties (20, 46). The global market for carotenoids used as food colorants and nutritional supplements was estimated at approximately $935 million in 2005 (11). More than 95% of all natural carotenoids are based on a symmetric C_{40} phytoene backbone, and only a small number of C_{30} and even fewer C_{50} carotenoids have been discovered (42).

C_{50} carotenoids have multiple conjugated double bonds, and they contain at least one hydroxyl group; both these features contribute to strong antioxidative properties (17, 30, 32, 38). In nature, C_{50} carotenoids are synthesized by bacteria of the order *Actinomycetales*, and to date, only two different C_{50} carotenoid biosynthetic pathways have been described in the literature. The biosynthetic pathways of the ε-cyclic C_{50} carotenoid decaprenoxanthin [2,2′-bis-(4-hydroxy-3-methybut-2-enyl)-ε,ε-carotene] and the β-cyclic C_{50} carotenoid C.p.450 [2,2′-bis-(4-hydroxy-3-methybut-2-enyl)-β,β-carotene] have been elucidated in *Corynebacterium glutamicum* (22, 23) and in *Dietzia* sp. CQ4 (41), respectively. For both pathways, the common precursor, C_{40} lycopene, is synthesized from C_{15} farnesyl pyrophosphate (FPP) via the methylerythritol 4-phosphate (MEP) pathway, which is present in most eubacteria (33). Effective lycopene production has been achieved in genetically engineered noncarotenogenic hosts, such as *Escherichia coli* and *Saccharomyces cerevisiae* (9). Accordingly, the potential of using such biotechnologically relevant hosts for heterologous production of any lycopene-derived carotenoids has generated high interest.

The biosynthesis of cyclic C_{50} carotenoids from lycopene is catalyzed by lycopene elongase and carotenoid cyclases. Even though most carotenoids in plants and microorganisms exhibit cyclic structures, cyclization reactions were predominantly known for C_{40} pathways (45) catalyzed by monomeric enzymes that have been isolated from plants and bacteria (5, 16, 27, 29, 31, 36). In *C. glutamicum*, the genes *crtYe*, *crtYf*, and *crtEb* were identified as being involved in the conversion of lycopene to the ε-cyclic C_{50} carotenoid decaprenoxanthin (22, 44). Sequential elongation of lycopene into the acyclic C_{50} carotenoid flavuxanthin was catalyzed by the *crtEb* gene product lycopene elongase. Subsequent cyclization to decaprenoxanthin was cat-

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Source
Strains		
E. coli DH5α	General cloning host	Gibco-BRL
E. coli XL1-Blue	Used as host for heterologous carotenoid production	Stratagene
M. luteus NCTC2665	Wild-type strain	NCTC
M. luteus Otnes7	Marine wild-type isolate	This work
C. glutamicum MJ-233C-MV10	Tn*31831* mutant of *C. glutamicum* MJ-233C; contains a wild-type *crt* gene cluster	44
Plasmids		
pGEM-T	Amp^r; standard cloning vector	Promega
pJBphOx	Amp^r; pJB658 derivative with inducible *Pm-xylS* promoter/regulator system	37
pAC-LYC	Cm^r; lycopene-producing plasmid containing *crtEIB* from *P. ananatis*; p15A *ori*	8
pCRT-EBIE2YgYh-2665	pJBphOx with *scFv-phOx* gene replaced with the c*rtEBIE2YgYh* region from strain Otnes7	This work
pCRT-EBI-2665	pJBphOx with *scFv-phOx* gene replaced with *crtEBI* from strain NCTC 2665	This work
pCRT-E2YgYh-O7	pJBphOx with *scFv-phOx* gene replaced with *crtE2YgYh* from strain Otnes7	This work
pCRT-E2YgYh-2665	pJBphOx with *scFv-phOx* gene replaced with *crtE2YgYh* from strain NCTC 2665	This work
pCRT-E2Yg-O7	pJBphOx with *scFv-phOx* gene replaced with *crtE2Yg* from strain Otnes7	This work
pCRT-E2Yg-2665	pJBphOx with *scFv-phOx* gene replaced with *crtE2Yg* from strain NCTC2665	This work
pCRT-E2-O7	pJBphOx with *scFv-phOx* gene replaced with *crtE2* from strain Otnes7	This work
pCRT-E2-2665	pJBphOx with *scFv-phOx* gene replaced with *crtE2* from strain NCTC2665	This work
pCRT-YgYh-O7	pJBphOx with *scFv-phOx* gene replaced with *crtYgYh* from strain Otnes7	This work
pCRT-YgYh-2665	pJBphOx with *scFv-phOx* gene replaced with *crtYgYh* from strain NCTC2665	This work
pCRT-E2YgYhX-O7	pJBphOx with *scFv-phOx* gene replaced with *crtE2YgYhX* from strain Otnes7	This work
pCRT-E2-O7-YeYf-MJ	pJBphOx with *scFv-phOx* gene replaced with *crtE2* from strain Otnes7 and *YeYf* from *C. glutamicum*	This work
pCRT-YeYfEb-MJ	pJBphOx with *scFv-phOx* gene replaced with *crtYeYfEb* from *C. glutamicum*	This work
pCRT-E2Yg-2665-Yf-MJ	pJBphOx with *scFv-phOx* gene substituted with *crtE2Yg* from strain Otnes7 and *crtYf* from *C. glutamicum*	This work

[a] Amp^r, ampicillin resistance; Cm^r, chloramphenicol resistance.

alyzed by a heterodimeric C_{50} carotenoid, ε-cyclase, encoded by *crtYe* and *crtYf* (22). *C. glutamicum* can synthesize both mono- and diglucosylated decaprenoxanthin; however, the genetic and enzymatic bases for glucosylation of decaprenoxanthin are unknown. Analogous to decaprenoxanthin, biosynthesis of the β-cyclic C_{50} carotenoid C.p.450 in *Dietzia* sp. CQ4 from lycopene involves lycopene elongase and C_{50} carotenoid β-cyclase activities (41).

While most cyclic carotenoids exhibit β-rings, ε-ring-containing pigments are common in higher plants (7), and carotenoids substituted only with γ-rings are rarely observed in plants and algae (14). To date, no biosynthetic pathway for γ-cyclic C_{50} carotenoids has been reported in the literature.

Micrococcus luteus NCTC2665 (the "Fleming strain") is a Gram-positive bacterium belonging to the family *Micrococcaceae* within the order *Actinomycetales*. The carotenoids, including the γ-cyclic C_{50} sarcinaxanthin [(2*R*,6*R*,2′*R*,6′*R*)-(2,2′-bis(4-hydroxy-3-methyl-2-butenyl)-γ,γ-carotene)], synthesized by this bacterium have been identified and structurally elucidated (26). We recently isolated and characterized several wild-type *M. luteus* strains from the sea surface microlayer of the middle part of the Norwegian coast (39). Here, we report one additional such marine *M. luteus* isolate, designated Otnes7, forming color-intensive colonies indicating high sarcinaxanthin production levels. Both Otnes7 and NCTC2665 were used as *M. luteus* model strains, and the sarcinaxanthin biosynthetic gene clusters were cloned from both strains. The complete sarcinaxanthin biosynthetic pathway from lycopene was elucidated, including glucosylation, and we also explored the potential of using Otnes7-derived genes to achieve effective heterologous production of sarcinaxanthin in *E. coli*. The results add important new knowledge of the biosynthesis of C_{50} carotenoids, and in particular, they highlight the diverse functions of C_{50} carotenoid cyclases leading to synthesis of structurally different carotenoids.

MATERIALS AND METHODS

Bacteria, plasmids, standard DNA manipulations, and growth media. The bacterial strains and plasmids used in this work are listed in Table 1. Bacteria were cultivated in Luria-Bertani (LB) broth (35), and recombinant *E. coli* cultures were supplemented with ampicillin (100 μg/ml) and chloramphenicol (30 μg/ml) as appropriate. *M. luteus* and *C. glutamicum* strains were grown at 30°C and 225-rpm agitation for 24 h, and *E. coli* strains for cloning purposes were grown at 37°C and 225-rpm agitation overnight. For heterologous production of carotenoids, overnight cultures (100 ml) of recombinant *E. coli* cells grown at 30°C with 180-rpm agitation in shake flasks (500 ml) were diluted 1% in prewarmed medium with 0.5 mM of the *Pm* promoter inducer *m*-toluic acid (37) added unless otherwise indicated. In order to elucidate maximal sarcinaxanthin production yields, samples were initially taken after 16 h, 24 h, and 48 h and analyzed quantitatively as described below. The highest carotenoid abundance was typically observed after 48 h of cultivation, but it was only marginally higher than after 24 h of cultivation (data not shown). This is in agreement with analogous reports for heterologous production of zeaxanthin in *E. coli* (34), and therefore, production analyses were routinely performed by analyzing samples collected after 48 h. Standard DNA manipulations were performed according to the method of Sambrook et al. (35), and isolation of total DNA from *M. luteus* strains was performed as described previously (43).

Vector construction. (i) pCRT-EBIE2YgYh-2665 and pCRT-EBI-2665. The complete *crtEBIE2YgYh* gene cluster of *M. luteus* NCTC2665 was PCR amplified from genomic DNA by using the primer pair crtE-F (5′-TTTTT**CATATG**GGTGAAGCGAGGACGGG-3′) and crtYh-R (5′-TTTTT**GCGGCCGC**TCAGCGATCGTCCGGGTGGGG-3′). The *crtEBI* region of *M. luteus* NCTC2665 was PCR amplified from genomic DNA by using the primer pair crtE-F (see above) and crtI-R (5′-TTTTT**GCGGCCGC**TCATGTGCCGCTCCCCCCGG). The re-

sulting PCR products (5,283 bp and 3,693 bp, respectively) were end digested with NdeI and NotI (the recognition sites are indicated in boldface in the primer sequences) and ligated into the corresponding sites of pJBphOx (37), yielding plasmids pCRT-EBIE2YgYh-2665 and pCRT-EBI-2665, respectively.

(ii) pCRT-E2YgYh-2665 and pCRT-E2YgYh-O7. The *crtE2YgYh* regions of *M. luteus* strains NCTC2665 and Otnes7 were PCR amplified from genomic DNA using primers crtE2-F (5′-TTTTT**CATATG**ATCCGCACCCTCTTCTG-3′) and crtYh-R (see above). The PCR products obtained (1,615 bp and 1,618 bp, respectively) were blunt-end ligated into the pGEM-T vector system (Promega, Madison, WI). The resulting plasmids were digested with NdeI and NotI (the recognition site is indicated in boldface in the primer), and the inserts were ligated into the corresponding sites of pJBphOx, yielding plasmids pCRT-E2YgYh-2665 and pCRT-E2YgYh-O7, respectively.

(iii) pCRT-E2YgYhX-O7. The *crtE2YgYhX* region of *M. luteus* strain Otnes7 was PCR amplified from genomic DNA using primers crtE2-F (see above) and crtYX-R (5′-TTTTT**CCTAGG**AGATGGCCGCGAACATCCTG). In the resulting PCR product, *crtYh* and *crtX* were separated by or1008, encoding a putative protein with no assigned function. The PCR product was end digested with NdeI and BlnI (the recognition site is indicated in boldface in the primer), and the 3,085-bp fragment was ligated into the corresponding sites of pJBphOx, resulting in plasmid pCRT-E2YgYhX-O7.

(iv) pCRT-E2Yg-O7 and pCRT-E2Yg-2665. The *crtE2Yg* coding regions of *M. luteus* strains NCTC2665 and Otnes7 were PCR amplified from chromosomal DNA using primers crtE2-F (see above) and crtYg-R (5′-TTTTT**GCGGCCGC**TCACCGGCTCCCCCGGTCGGTC-3′). The PCR products obtained were end digested with NdeI and NotI (the recognition site is indicated in boldface in the primer sequence), and the resulting 1,247-bp fragments were ligated into the corresponding sites of pJBphOx, resulting in plasmids pCRT-E2Yg-2665 and pCRT-E2Yg-O7, respectively.

(v) pCRT-E2-O7 and pCRT-E2-2665. The *crtE2* genes of *M. luteus* strains NCTC2665 and Otnes7 were PCR amplified from chromosomal DNA using primers crtE2-F (see above) and crtE2-R (5′-TTTTT**GCGGCCGC**TCATGCCGCCGCCCCCCGGG-3′). The resulting PCR products were end digested with NdeI and NotI (the recognition site is indicated in boldface in the primer sequence), and the 890-bp fragments were ligated into the corresponding sites of pJBphOx, resulting in plasmids pCRT-E2-2665 and pCRT-E2-O7, respectively.

(vi) pCRT-YgYh-O7 and pCRT-YgYh-2665. The *crtYgYh* regions of *M. luteus* strains NCTC2665 and Otnes7 were PCR amplified from genomic DNA by using primers crtYg-F (5′-TTTTT**CATATG**ATCTACCTGCTGGCCCT-3′) and crtYh-R (see above). The resulting 734-bp PCR products were end digested with NdeI (the restriction site is indicated in boldface in the primer sequence) and NotI and ligated into the corresponding sites of pJBphOx, resulting in plasmids pCRT-YgYh-2665 and pCRT-YgYh-O7, respectively.

(vii) pCRT-E2-O7-YeYf-MJ. According to the gene sequences of *crtE2* in *M. luteus* Otnes7 and *crtYeYf* in *C. glutamicum* MJ233-MV10, four primers, crtE2-F (5′-TGACCAACG**ACCGGT**AGCGGAG-3′) and crtE2-i-R (5′-*CCCATCCACTAAACTTAAAC*ATCATGCCGCCGCCCCCCGG-3′), and crtYe-i-F (5′-*TGTTTAAGTTTAGTGGATGGG*TTGATCCCTATCATCGATATTTCAC-3′) and crtYf-R (5′-TTTT**GCGGCCGC**TTTTCCATCATGACTACGGCTTTTC), were used. Primers crtE2-i-R and crtYe-i-F contained homologous extensions of 21 bp (italics) at the 5′ ends as linker sequences in order to allow crossover PCR. The primer pair crtE2-F and crtE2-i-R was used to amplify a 1,227-bp fragment containing the *crtE2* gene from genomic *M. luteus* DNA, and the primer pair crtYe-i-F and crtYf-R was used to amplify an 885-bp *crtYeYf*-containing fragment from genomic DNA of *C. glutamicum* MJ-233C-MV10. The resulting PCR fragments were used as templates for PCR with the primer pair crtE2-F and crtYe-R to amplify a hybrid DNA fragment (2,090 bp) containing *crtE2* from *M. luteus* and *crtYeYf* from *C. glutamicum* connected by the 21-bp linker sequence. The resulting hybrid fragment was end digested with AgeI and NotI (the restriction site is indicated in boldface in the primer sequence), and the 2,070-bp fragment obtained was ligated into the corresponding sites of pJB658phOx, resulting in plasmid pCRT-E2-O7-YeYf-MJ.

(viii) pCRT-YeYfEb-MJ. The *crtYeYfEb* genes from *C. glutamicum* strain MJ-233C-MV10 (Table 1) were PCR amplified from genomic DNA using primers crtYe-F1 (5′-TGGCTATC**TCTAGA**AAGGCCTACCCCTTAGGCTTTATGCAACAGAAACAATAATAATGGAGTCATGAACATATGATCCCTATCATCGATATTTCAC-3′) and crtYf-R (5′-TTTT**GCGGCCGC**CTGATCGGATAAAAGCAGAGTTATATC-3′). The resulting PCR product was digested with XbaI and NotI (the restriction site is indicated in boldface in the primer sequence), and the 1,789-bp fragment was ligated into the corresponding sites of pJBphOx, resulting in plasmid pCRT-YeYfEb-MJ.

All the constructed vectors were verified by DNA sequencing and transformed by electroporation (10) into the production host strains *E. coli* XL1-Blue and the lycopene-producing *E. coli* XL1-Blue(pAC-LYC) (8).

Extraction of carotenoids from bacterial-cell cultures. To extract carotenoids from *M. luteus* strains, cells were harvested, washed with deionized H_2O, and treated with lysozyme (20 mg/ml) and lipase (Fluka Chemicals, Germany) according to the method of Kaiser et al. (18), and the pigments were extracted with a mixture (7:3) of methanol and acetone. For recombinant *E. coli* strains, 50-ml aliquots of the cell cultures were centrifuged at 10,000 × *g* for 3 min, and the pellets were washed with deionized H_2O; the cells were then frozen and thawed to facilitate extraction. Finally the pigments were extracted with 4 ml methanol-acetone (7:3) at 55°C for 15 min with thorough vortexing every 5 min. When necessary, up to three extraction cycles were performed to remove all visible colors from the cell pellet. When selective extraction for xanthophylls was desired, pure methanol was used. Butyhydroxytoluene (BHT) (0.05%) was added to the organic solvent to contribute to the stabilization of carotenoids (18). Samples for preparative high-performance liquid chromatography (HPLC) were in addition partitioned into 50% diethyl ether in petroleum ether. The collected upper phase was evaporated to dryness and dissolved in methanol.

Quantitative and qualitative LC-MS analyses of carotenoids in cell extracts. Liquid chromatography-mass spectrometry (LC-MS) analyses of carotenoid-containing extracts were performed on an Agilent Ion Trap SL mass spectrometer equipped in front with an Agilent 1100 series HPLC system, including a diode array detector (DAD) for UV/visible (Vis) spectrum recording. Quantification of carotenoids was performed using the extracted wavelength chromatogram at peak λ_{max}, 450 ± 16 nm for sarcinaxanthin and carotenoids with corresponding UV/Vis profiles and 470 ± 16 nm for lycopene and corresponding carotenoids, while MS detection was used to confirm the identities of known peaks for quantification and to determine the molecular masses of unknown carotenoids in the various cell extracts. Trans-beta-apo-8′-carotenal (Sigma) and lycopene (Fluka) were used as standards. They were dissolved in chloroform according to their solubilities and diluted in methanol. The correct concentrations of the prepared standard solutions were calculated from absorbance measurements of the solutions and by using the specific extinction coefficients $E^{1\%}{}_{1cm}$, i.e., the absorption of 1% solution in a 1-cm cuvette at the maximum absorption wavelength, of 3,450 for lycopene and 2,590 for apocarotenal (15, 18). The standards were filtered through a syringe 0.2-μm polypropylene filter (Pall Gelman) and stored in amber glass vessels at −80°C under an N_2 atmosphere if not used immediately.

Two HPLC protocols were used, a fast, high-throughput method for quantification of known carotenoids and a slow method with higher resolution for qualitative detection of all carotenoids in an extract permitting determination of the UV/Vis spectra and molecular masses of unknown carotenoids. A 2.1- by 30-mm Zorbax RR SB RP C_{18} column was used for the fast, high-throughput method. The carotenoids were eluted isocratically in methanol for 5 min. The column flow was kept at 0.4 ml/min, and 10 μl extract was injected for each run. The slow run method was run isocratically for 25 min with a mobile phase composition of MeOH/acetonitrile (7:3) with a 2.1- by 150-mm Zorbax SB RP C_{18} column using a flow rate of 250 μl/min. Ten or 20 μl extract was injected depending on the concentrations of carotenoids in the various extracts. The mass spectrometer was operated in positive scan mode using chemical ionization. The settings of the atmospheric pressure chemical ionization (APCI) source were 325°C dry temperature, 350°C vaporizer temperature, 50 lb/in^2 nebulizer pressure, and 5.0 liter/min dry gas.

Purification of carotenoids. For purification of carotenoids, preparative HPLC was performed on an Agilent preparative HPLC 1100 series system equipped with two preparative HPLC pumps, a preparative autosampler, and a preparative fraction collector. The mobile phases were methanol in channel 1 and acetonitrile in channel 2. Samples (2 ml) were injected at a flow rate of 20 ml/min into a Zorbax RP C_{18} 21- by 250-mm preparative LC column. Online MS analysis was performed by splitting the flow 1:200 after the column using an Agilent LC flow splitter, and a makeup flow of 1 ml methanol/min was used to carry the analytes to the mass spectrometer with less than a 15-s delay. The diode array detector was used to trigger fraction collection.

Carotenoid structure determination by NMR. When appropriate, nuclear magnetic resonance (NMR) was used for carotenoid structure determination. All NMR spectra were recorded on a Bruker Avance 600-MHz instrument fitted with an inverse triple resonance cryoprobe (TCI) using $CDCl_3$ as a solvent with trimethylsilyl (TMS) as an internal reference. ^1H and ^{13}C signals were unambiguously assigned with the aid of in-phase correlation spectroscopy (ip-SCOPY), heteronuclear single-quantum coherence (HSQC), heteronuclear multiple-bond correlation (HMBC), nuclear Overhauser effect spectroscopy (NOESY), and HSQC–total-correlation spectroscopy (TOCSY) experiments.

RESULTS

Analysis of carotenoids produced by *M. luteus* NCTC2665 and a new marine *M. luteus* isolate designated Otnes7. As a basis for the current studies, we characterized the major carotenoids synthesized by the *M. luteus* wild-type strain NCTC2665. In addition, we report here one new selected marine isolate, designated Otnes7, classified as an *M. luteus* strain by 16S rRNA sequence analysis (performed by NCIMB Ltd., Scotland) and forming colonies on LB agar plates with higher color intensity than other wild-type *M. luteus* strains (data not shown). We chose to include Otnes7 as an alternative model strain in this study, in particular to investigate whether its genes might also be favorable for efficient heterologous sarcinaxanthin production in *E. coli* strains (see below).

Cell extracts from shake flask cultures of strain NCTC2665 were analyzed by LC-MS, and one major peak (peak 3) (Fig. 1A) was identical to that of sarcinaxanthin purified and structurally identified by NMR earlier from wild-type *M. luteus* strains (39). In addition, two minor peaks, peak 1 and peak 2, were identified, with the same absorption spectra as sarcinaxanthin (Fig. 1A). The retention time of peak 2 was equal to that of sarcinaxanthin monoglucoside identified by NMR earlier (39), while peak 1 was more polar and therefore was predicted to represent sarcinaxanthin diglucoside (Table 2). An analogous analysis of strain Otnes7 revealed that it produced the same type of carotenoids; however, the total carotenoid level was higher (190 μg/g cell dry weight [CDW]) than that of NCTC2665 cells (145 μg/g CDW) under the conditions tested, which was in agreement with the different colony color intensities of the two strains.

Genetic characterization and heterologous expression of the *M. luteus* sarcinaxanthin biosynthetic gene cluster. The genome sequence of *M. luteus* strain NCTC2665 (accession number NC_012803) has been deposited in the databases, and just before the submission of this paper, an accompanying publication appeared in the scientific literature (47). *In silico* screening of the sequence data resulted in the identification of a putative carotenoid biosynthesis gene cluster consisting of a total of nine open reading frames, or1007 to or1015. The genetic organization of the carotenoid (*crt*) genes in *M. luteus* displayed certain similarities to the previously published biosynthetic gene clusters for the C_{50} carotenoids C.p.450 and decaprenoxanthin in *Dietzia* sp. (41) and *C. glutamicum* (22), respectively (Fig. 2). The deduced *M. luteus* gene products displayed between 27% and 55% primary sequence identity to enzymes of the decaprenoxanthin and C.p.450 biosynthetic pathways. Based on these sequence analyses, the *M. luteus* genes *crtE* (encoding geranyl geranyl pyrophosphate [GGPP] synthase), *crtB* (encoding phytoene synthase), *crtI* (encoding phytoene desaturase), *crtE2* (encoding lycopene elongase), *crtYg* (encoding the C_{50} cyclase subunit), *crtYh* (encoding the C_{50} cyclase subunit), and *crtX* (encoding glycosyl transferase) were assigned (Table 3). In addition, or1008 and or1015 encoded putative proteins with no assigned functions. In an attempt to identify relevant transcription initiation elements, the "neural network promoter prediction" method (http://www.fruitfly.org/seq_tools/promoter.html) was applied to the entire *crt* gene cluster, including or1015 and the upstream region (Fig. 2). By far the highest score was observed for a nucleotide sequence located 112 to 62 bp upstream of the *crtE* start codon, suggesting that the gene cluster is transcribed as a polycistronic operon from this promoter.

To experimentally verify that the identified *M. luteus* gene cluster encoded an active sarcinaxanthin biosynthetic pathway, the entire *crtEBIE2YgYh* region from NCTC2665 was cloned in frame and under the transcriptional control of the positively regulated *Pm-xylS* promoter/regulator system in plasmid pJBphOx (37). This expression vector has many favorable properties useful for regulated expression of genes and pathways at relevant levels in Gram-negative bacteria (1). The resulting plasmid, pCRT-EBIE2YgYh-2665 (Table 1), was transformed into the noncarotenogenic *E. coli* host strain XL1-Blue, and the recombinant strain was analyzed for carotenoid production under *Pm*-induced conditions (0.5 mM *m*-toluic acid) over 48 h (see Materials and Methods). LC-MS analysis of cell extracts revealed a small peak at a retention time, absorption spectrum, and molecular mass identical to those of sarcinaxanthin identified in the *M. luteus* strains (see above). The recombinant *E. coli* strain produced small amounts of sarcinaxanthin (10 to 15 μg/g CDW). No sarcinaxanthin was detected in plasmid-free cells, thus confirming that the identified gene cluster encodes a sarcinaxanthin biosynthetic pathway from farnesyl pyrophosphate. The biological role of the *crtX* gene was experimentally confirmed (see below).

Sarcinaxanthin production levels in *E. coli* can be increased 150-fold (2.5 mg/g CDW) by expressing Otnes7-derived *crtE2YgYh* genes in a lycopene-producing host. To investigate the reason for the poor sarcinaxanthin production levels obtained in *E. coli*, we established a recombinant strain, *E. coli*(pCRT-EBI-2665), expressing the *crtE*, *crtB*, and *crtI* genes from NCTC2665, encoding enzymes assumed to catalyze the conversion of FPP into lycopene (Fig. 2 and Table 3). Analysis of this recombinant strain under *Pm*-induced conditions (0.5 mM toluic acid) confirmed that it produced lycopene as the sole carotenoid. However, the lycopene production yield was low (8 to 12 μg/g CDW) and in the same range as the sarcinaxanthin production yield obtained when the complete *crtEBIE2YgYh* gene cluster was expressed (see above). These data suggested that lycopene synthesis might be a bottleneck for efficient sarcinaxanthin production in this *E. coli* host. Therefore, *E. coli* XL1-Blue was transformed with plasmid pAC-LYC (6, 8) harboring the *Pantoea ananatis crtEIB* genes encoding three enzymes for biosynthesis of lycopene from isoprenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP). LC-MS analysis confirmed that the resulting strain, *E. coli*(pAC-LYC), accumulated lycopene (1.8 mg/g CDW) as the sole carotenoid, and therefore, further carotenoid production experiments were performed using XL1-Blue(pAC-LYC) as a host.

We then established *E. coli*(pAC-LYC)(pCRT-E2YgYh-2665) expressing the *crtE2*, *crtYg*, and *crtYh* genes from NCTC2665 (Table 1). LC-MS analysis of cell extracts revealed a total maximum carotenoid accumulation of 2.3 mg/g CDW, and about 98% of the total carotenoid produced was identified as sarcinaxanthin (Fig. 3, 0.5 mM inducer). These data demonstrated that the *M. luteus* NCTC2665 *crtE2YgYh* gene products could effectively convert lycopene into sarcinaxanthin. This result also confirmed that *M. luteus* genes were not effi-

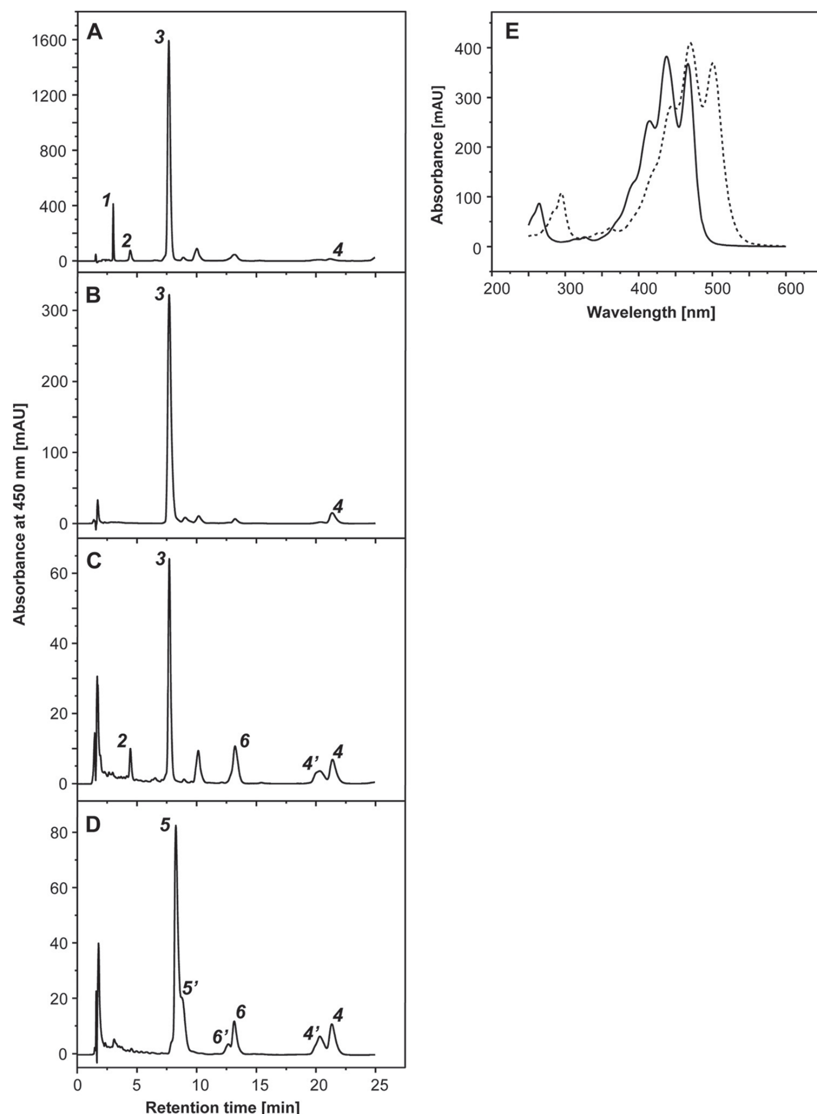

FIG. 1. (A to D) HPLC elution profiles of carotenoids extracted from *M. luteus* strains Otnes7 (A), *E. coli*(pAC-LYC)(pCRT-E2YgYh-O7) (B), *E. coli*(pAC-LYC)(pCRT-E2YgYhX-O7) (C), and *E. coli*(pAC-LYC)(pCRT-E2-O7) (D). Peak 1, sarcinaxanthin diglucoside; peak 2, sarcinaxanthin monoglucoside; peak 3, sarcinaxanthin; peak 4, lycopene; peak 5, flavuxanthin; peak 6, nonaflavuxanthin; Peaks 4′, 5′, and 6′ are the *cis* isomers of 4, 5, and 6, respectively. (E) Absorption spectra of carotenoids from peaks 1, 2, and 3 (solid line) and peaks 4, 5, and 6 (dashed line). AU, arbitrary units.

cient for lycopene production under the conditions tested. The reason for this is unknown, and it was not further investigated in the present study.

It was of interest to test if Otnes7 genes could contribute to more efficient sarcinaxanthin production levels in this host. Therefore, the alternative strain *E. coli*(pAC-LYC)(pCRT-E2YgYh-O7) expressing the *crtE2*, *crtYg*, and *crtYh* genes from Otnes7 was established. The total carotenoid production level (2.5 mg/g CDW) of the resulting recombinant strain was slightly higher than that of the analogous strain *E. coli*(pAC-LYC)(pCRT-E2YgYh-2665) (2.3 mg/g CDW). Interestingly, we noticed that all (100%) of the total carotenoid produced by *E. coli*(pAC-LYC)(pCRT-E2YgYh-O7) was sarcinaxanthin and no lycopene was present in these cells (Fig. 3). This demonstrated very efficient lycopene conversion by using Otnes7-derived genes, indicating that lycopene synthesis is a bottle-

TABLE 2. Characteristics of carotenoids extracted from *M. luteus* strain Otnes7 and from recombinant *E. coli* strains[a]

Carotenoid (trivial name)	λ_{max} (nm) in the HPLC eluent	Molecular mass (Da)	Retention time (min)
Sarcinaxanthin diglucoside	414 438 467	1,028	3.0
Sarcinaxanthin monoglucoside	414 438 467	886	4.5
Sarcinaxanthin	414 438 467	704	7.7
Flavuxanthin	445 470 501	704	8.2
Nonaflavuxanthin	445 470 501	620	13.2
Lycopene	445 470 501	536	21.3
Decaprenoxanthin	414 438 467	704	10.1
Sarprenoxanthin	414 438 467	704	8.9

[a] The carotenoids were dissolved in methanol and separated by HPLC using the Zorbax C_{18} 150- by 30-mm column (see Materials and Methods). All these extracted carotenoids have a characteristic three-peak absorption profile (Fig. 1E), and all λ_{max} values are given.

neck for further improved sarcinaxanthin production under the conditions tested. Therefore, to further compare the efficiency of using Otnes7- versus NCTC2665-derived biosynthetic genes, production analyses were performed with different *Pm* inducer concentrations (Fig. 3). The results demonstrated that the strain *E. coli*(pAC-LYC)(pCRT-E2YgYh-O7) produced sarcinaxanthin at higher levels than *E. coli*(pAC-LYC)(pCRT-E2YgYh-2665) under all induction conditions tested, thus confirming that Otnes7 genes may be preferable for efficient sarcinaxanthin production in the *E. coli* host. DNA sequence analysis of the cloned Otnes7 *crtE2YgYh* fragment revealed a total of 24 nucleotide substitutions compared to the corresponding NTCT2665 DNA sequence, corresponding to four amino acid substitutions in CrtE2, five in CrtYg, and two substitutions plus one insertion in CrtYh. Whether these sequence variations positively affect the expression levels or the catalytic properties of the respective proteins remains unknown, and it was not further investigated here.

The *M. luteus crtE2* gene product catalyzes *in vivo* conversion of lycopene to C_{45} nonaflavuxanthin and C_{50} flavuxanthin. To elucidate the individual biosynthetic steps in the conversion of lycopene to sarcinaxanthin, we established strain *E. coli*(pAC-LYC)(pCRT-E2-2665) expressing the *crtE2* genes from NCTC2665 (Table 1) and analyzed it for carotenoid production. Two different carotenoids were accumulated in the cells in addition to lycopene (Fig. 1D); all three compounds shared identical UV/Vis profiles. No sarcinaxanthin was detected. The minor carotenoid had a molecular mass of 620 Da, indicating a C_{45} xanthophyll compared with reported values (620 to 624 Da) (3), and the major carotenoid had a molecular mass of 704 Da, indicating a C_{50} xanthophyll (704 to 738 Da) (3). The major carotenoid was purified by preparative HPLC and analyzed by NMR (see Table S1 in the supplemental material). Inspection of ^{1}H, ^{13}C, and HSQC spectra revealed chemical shifts in agreement with reported data for the acyclic C_{50} carotenoid flavuxanthin (24). The minor carotenoid was identified as nonaflavuxanthin on the basis of the UV/Vis profile and the mass (Table 2). These results verified that the *M. luteus crtE2* gene encodes a lycopene elongase catalyzing the sequential elongation of the C_{40} carotenoid lycopene via the C_{45} carotenoid nonaflavuxanthin to the C_{50} carotenoid flavuxanthin. A similar analysis using the analogous strain *E. coli*(pAC-LYC)(pCRT-E2-Otnes7) gave the same conclusion (data not shown). We noticed that the relative conversion of lycopene was substantially higher in the latter strain (79% versus 23%), which was in agreement with the generally effective sarcinaxanthin production obtained when Otnes7 genes were expressed (Fig. 3).

The *M. luteus crtYg* and *crtYh* genes together encode an active C_{50} carotenoid cyclase catalyzing cyclization of C_{50} flavuxanthin to C_{50} sarcinaxanthin *in vivo*. To investigate if *crtYg* encoded any cyclase activity, we constructed and analyzed the recombinant strains *E. coli*(pAC-LYC)(pCRT-E2Yg-O7) and *E. coli*(pAC-LYC)(pCRT-E2Yg-2665) expressing the *crtE* and *crtYg* genes from NCTC2665 and Otnes7, respectively (Table 1). The carotenoids produced by both strains were flavuxanthin, nonaflavuxanthin, and lycopene, and their relative abundances were similar to those in strains *E. coli*(pAC-LYC)(pCRT-E2-O7) and *E. coli*(pAC-LYC)(pCRT-E2-2665), respectively. No sarcinaxanthin was detected in either of the strains. These data thus implied that the CrtYg and CrtYh polypeptides must function together as an active carotenoid cyclase catalyzing cyclization of flavuxanthin to sarcinaxanthin *in vivo*. To analyze the specificity of this carotenoid cyclase, we established the recombinant strains *E. coli*(pAC-LYC)(pCRT-YgYh-O7) and *E. coli*(pAC-LYC)(pCRT-YgYh-2665) expressing the *crtYg* and *crtYh* genes from NCTC2665 and Otnes7, respectively (Table 1). The aim was to analyze whether the CrtYgYh cyclases could catalyze cyclization of lycopene *in vivo*. HPLC analysis showed that both strains accumulated lycopene as

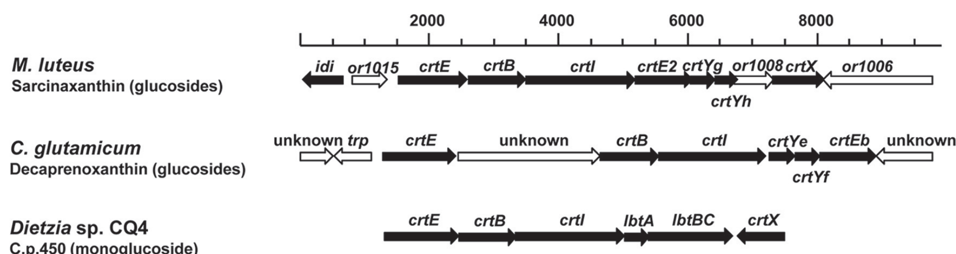

FIG. 2. Chromosomal organization of the *M. luteus* sarcinaxanthin biosynthetic gene cluster presented in this study. The analogous *C. glutamicum* and *Dietzia* sp. biosynthetic gene clusters for the C_{50} carotenoids decaprenoxanthin and C.p.450, respectively, are included for comparison. Genes indicated by white arrows are suggested not to be involved in carotenoid biosynthesis.

TABLE 3. *M. luteus* sarcinaxanthin biosynthetic genes and primary sequence comparison with respective homologues from biosynthesis of decaprenoxanthin and C.p.450 in *C. glutamicum* and *Dietzia* sp. CQ4, respectively

ORF[a]	Gene name	Predicted gene product	*C. glutamicum*		*Dietzia* sp. CQ4	
			Homologue	Primary sequence identity (%)	Homologue	Primary sequence identity (%)
or1007	*crtX*	Glycosyl transferase (CrtX)	None		CrtX	43
or1008		Unknown				
or1009	*crtYh*	C_{50} γ-cyclase subunit (CrtYh)	CrtYf	31	LbtBC[b]	38
or1010	*crtYg*	C_{50} γ-cyclase subunit (CrtYg)	CrtYe	32	LbtA	36
or1011	*crtE2*	Lycopene elongase (CrtE2)	CrtEb	50	LbtBC[c]	52
or1012	*crtI*	Phytoene desaturase (CrtI)	CrtI	43	CrtI	53
or1013	*crtB*	Phytoene synthase (CrtB)	CrtB	41	CrtB	48
or1014	*crtE*	GGPP synthase (CrtE)	CrtE	31	CrtE	33

[a] ORF, open reading frame.
[b] The N-terminal region of LbtBC (amino acids 1 to 134) is homologous to those of *M. luteus* CrtYh and *C. glutamicum* CrtYf, respectively.
[c] The C-terminal region of LbtBC (amino acids 135 to 432) is homologous to those of *M. luteus* CrtE2 and *C. glutamicum* CrtEb, respectively.

the only carotenoid (data not shown), confirming that the *crtYgYh* gene products cannot use lycopene as a substrate. These data confirmed that the CrtYg and CrtYh polypeptides together constitute an active γ-type C_{50} carotenoid cyclase catalyzing cyclization of flavuxanthin to sarcinaxanthin *in vivo*.

The *M. luteus crtX* gene encodes an active glycosyl transferase that can be used to produce monoglucosylated sarcinaxanthin in *E. coli*. Downstream of *crtYh* is or1008, encoding a putative protein with no significant homology to any known proteins, followed by or1007, which encodes a polypeptide sharing 46% primary sequence identity with the putative glucosyl transferase protein CrtX from *Dietzia* sp. CQ4 (41). To our knowledge, no analogous gene has been found in the *C. glutamicum* genome sequence, and yet this bacterium can synthesize glucosylated decaprenoxanthin (24). The or1007 gene is depicted here as *crtX* (Table 3), and to unravel its biological function, we constructed and analyzed the recombinant strain *E. coli*(pAC-LYC)(pCRT-E2YgYhX-O7), which expresses the *crtE2*, *crtYg*, *crtYh*, and *crtX* genes from Otnes7 (Table 1). The resulting HPLC profile (Fig. 1C) revealed sarcinaxanthin as the major carotenoid (peak 3), but an additional, more polar carotenoid

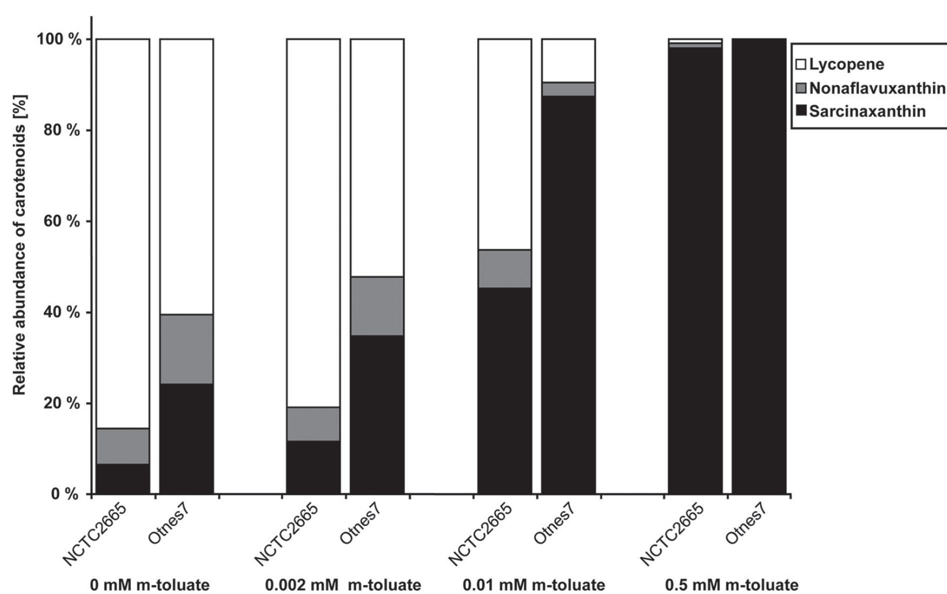

FIG. 3. Relative carotenoid abundances in extracts from *E. coli*(pAC-LYC)(pCRT-E2YgYh-O7) and *E. coli*(pAC-LYC)(pCRT-E2YgYh-2665) overexpressing *crtE2*, *crtYg*, and *crtYh* genes from *M. luteus* strains Otnes7 and NCTC2665 (Table 1) cultivated in the presence of various *Pm* inducer concentrations (0, 0.002, 0.01, and 0.5 mM *m*-toluic acid). The fractions of sarcinaxanthin, lycopene, and intermediates are indicated. Samples (three replicates) were analyzed after 48 h of cultivation to ensure maximum sarcinaxanthin production levels (see Materials and Methods).

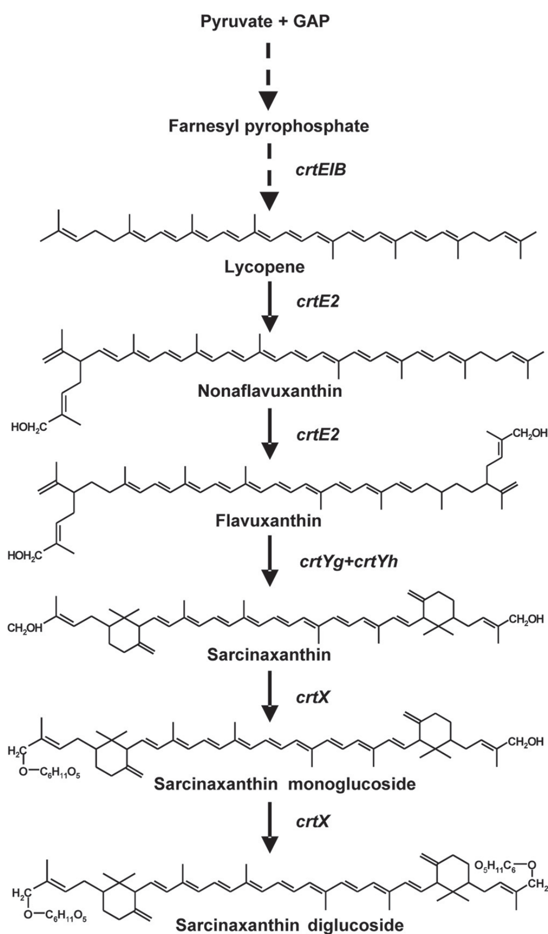

FIG. 4. Elucidated biosynthetic pathway for the individual steps in the formation of sarcinaxanthin and its glucosides from lycopene. CrtEBI, GGPP synthase, phytoene synthase, and phytoene desaturase; CrtE2, lycopene elongase; CrtYg plus CrtYf, C_{50} carotenoid γ-cyclase; CrtX, C_{50} carotenoid glycosyl transferase.

was eluted earlier (peak 2) and had a retention time and absorption spectrum identical to those of sarcinaxanthin monoglucoside from *M. luteus* Otnes 7 (Fig. 1C and E). Another minor peak was observed with the same retention time as sarcinaxanthin diglucoside produced by *M. luteus* strains (39); however, the amount detected was too small for a confident analysis of the mass and absorption spectrum. About 10% of the total sarcinaxanthin produced was glucosylated both in *M. luteus* wild-type strains and when heterologously produced in *E. coli* under the conditions tested. These results confirmed that *crtX* encodes an active glycosyl transferase that is necessary for the glucosylation of sarcinaxanthin. Based on all the accumulated data, we could deduce the complete biosynthetic pathway of sarcinaxanthin and its glucosides from FPP and via lycopene in *M. luteus*, as presented in Fig. 4.

Expression of a hybrid operon containing the decaprenoxanthin cyclase genes *crtYe* and *crtYf* leads to production of three different C_{50} carotenoids in *E. coli*. The biosynthetic pathway for decaprenoxanthin in *C. glutamicum* is reported to involve cyclization of flavuxanthin by an ε-cyclic C_{50} cyclase encoded by *crtYe* and *crtYf*. At this point, the biochemical functions of the C_{50} cyclase proteins in *M. luteus* and *C. glutamicum* seemed to represent the major difference between the biosynthetic pathways of sarcinaxanthin and decaprenoxanthin. To experimentally verify this, we established and analyzed *E. coli*(pAC-LYC)(pCRT-E2-O7-YeYf-MJ) expressing a hybrid operon containing *crtE2* from Otnes7 and *crtYe* and *crtYf* from *C. glutamicum* MJ233-MV10. We expected this recombinant strain to produce decaprenoxanthin. Surprisingly, LC-MS analysis revealed that three different cyclic C_{50} carotenoids were accumulated (Fig. 5), exhibiting the same UV/Vis absorbance spectrum and mass (Fig. 1E). The retention time for peak 1 was identical to that of sarcinaxanthin. Peaks 1, 2, and 3 represented 35%, 46%, and 19%, respectively, of the total carotenoid content of the recombinant cells. No lycopene was detected.

All three fractions were then purified by preparative HPLC and analyzed by NMR. The NMR data confirmed the identity of peak 1 as sarcinaxanthin (25), while peak 3 was identified as decaprenoxanthin (12, 13) (see Table S1 in the supplemental material). The NMR data for the purified peak 2 (Table 4), representing the major product, indicated a molecule that could not be clearly distinguished from a 1:1 mixture of sarcinaxanthin and decaprenoxanthin, so its existence had to be confirmed by chromatographic methods. The purified fractions were reanalyzed by LC using the slow run method (see Materials and Methods) to control its purity, and the corresponding chromatogram clearly shows that fraction 2 is a distinct carotenoid different from fractions 1 and 3 (Fig. 5B and C). Peak 2 was identified as a new bicyclic asymmetric C_{50} carotenoid with the systematic name 2,2′-bis(4-hydroxy-3-methyl-2-butenyl)-γ,ε-carotene. The compound exhibits one ε-cyclic and one γ-cyclic structure and thus appears to be a structural combination of sarcinaxanthin and decaprenoxanthin. This is in total agreement with the polarities of the three carotenoids (Fig. 5), and the compound was named sarprenoxanthin (Fig. 5 and 6). To our knowledge, this carotenoid has not been previously reported in the literature.

To rule out the possibility that these results were due to any unforeseen functions of the hybrid operon as such, we established the analogous strain *E. coli*(pAC-LYC)(pCRT-YeYfEb-MJ) expressing the decaprenoxanthin genes *crtYe*, *crtYf*, and *crtEb* from *C. glutamicum* (Fig. 2). The resulting chromatographic profile and absorbance spectrum were the same as in the hybrid construct, with relative abundance, mass, UV/Vis profile, and retention time identical to those of the carotenoids (data not shown). Moreover, DNA sequencing confirmed that the expressed *crt* genes were wild-type *C. glutamicum* sequences. Together, these results revealed that the *crtYe* and *crtYf* genes encoded a multifunctional C_{50} carotenoid cyclase that could catalyze the synthesis of three different bicyclic carotenoids. To our knowledge, this is the first report that *C. glutamicum* genes can be used to synthesize sarcinaxanthin and sarprenoxanthin.

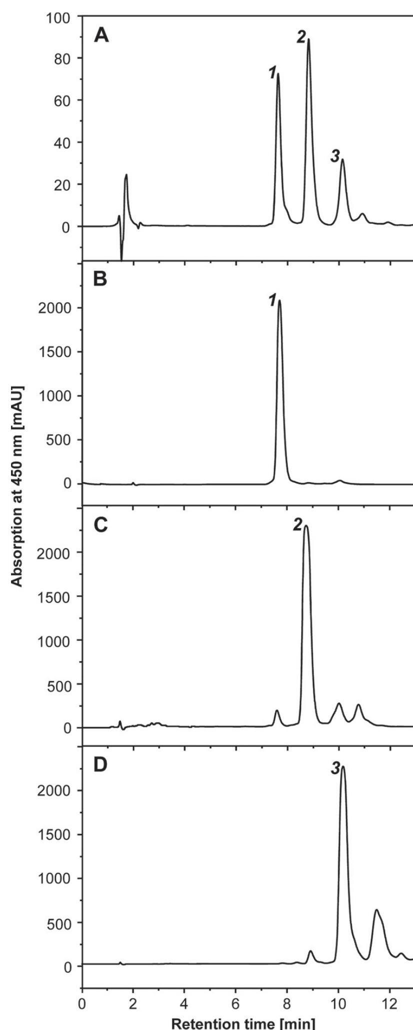

FIG. 5. HPLC elution profiles of the carotenoids extracted from *E. coli*(pAC-LYC)(pCRT-E2 ml-YeYfcg) (A); purified peak 1, sarcinaxanthin (B); peak 2, sarprenoxanthin (C); and peak 3, decaprenoxanthin (D).

DISCUSSION

In this paper, we report the cloning, characterization, and functional expression of the sarcinaxanthin biosynthetic gene cluster from *M. luteus* NCTC2665, consisting of six genes (*crtE*, *crtB*, *crtI*, *crtE2*, *crtYg*, and *crtYh*) encoding enzymes involved in the conversion of FPP into sarcinaxanthin. By expressing single genes and combinations of genes under the control of the positively regulated *Pm-xylS* promoter/regulator system in *E. coli* hosts, we discovered that sarcinaxanthin biosynthesis from the precursor FPP proceeds via lycopene, nonaflavuxanthin, and flavuxanthin to sarcinaxanthin (Fig. 4). *M. luteus* strains synthesized both mono- and diglucosylated derivatives of sarcinaxanthin, and we demonstrated that the *crtX* gene located proximal to the sarcinaxanthin genes encoded glycosyl transferase activity for the glucosylation of sarcinaxanthin (Fig. 2 and Table 3). No analogous glycosyl transferase-encoding gene has been identified in the natural decaprenoxanthin producer

TABLE 4. ^{1}H and ^{13}C NMR assignments of sarprenoxanthin[a]

Position	^{1}H	Multiplicity[b]	^{13}C
C-1			36.0
CH-2	1.36	m	44.2
CH$_2$-3	1.66	m	28.7
	2.07	m	
CH-4	5.45	m	121.4
C-5			134.4
CH-6	2.44	d (10.4)	56.6
CH-7	5.33	dd (15.4, 10.4)	130.3
CH-8	6.15	d (15.4)	138.1
C-9			135.3
CH-10	6.14	m	130.4
CH-11	6.62	m	124.9
CH-12	6.36	d (14.9)	137.4
C-13			136.4
CH-14	6.25	m	132.4
CH-15	6.63	m	130.0
Me-16	0.93	s; *trans* to C-7	26.8
Me-17	0.74	s; *cis* to C-7	16.3
Me-18	1.53	s	23.1
Me-19	1.93	s	13.16
Me-20	1.97	s	12.8
CH$_2$-1″	1.81	m	28.0
	2.27	m	
CH-2″	5.41	m	126.1
C-3″			135.2
CH$_2$-4″	4.01	s	69.2
Me-C-3″	1.67	s	13.8
C-1′			39.3
CH-2′	1.28	m	48.5
CH$_2$-3′	1.19	m	28.9
	1.73	m	
CH$_2$-4′	2.04	m	36.3
	2.35	ddd (13.4, 4.2, 2.6)	
C-5′			150.4
CH-6′	2.48	d (9.9)	58.5
CH-7′	5.83	dd (15.5, 9.9)	128.4
CH-8′	6.12	d (15.5)	137.6
C-9′			135.4
CH-10′	6.12	m	130.7
CH-11′	6.62	m	124.9
CH-12′	6.34	d (14.9)	137.4
C-13′			136.4
CH-14′	6.25	m	132.4
CH-15′	6.63	m	130.0
Me-16′	0.95	s; *trans* to C-7′	27.7
Me-17′	0.73	s; *cis* to C-7′	15.3
CH$_2$-18′	4.53	s; *cis* to C-6′	108.1
	4.76	s; *trans* to C-6′	
Me-19′	1.98	s	13.21
Me-20′	1.97	s	12.8
CH$_2$-1‴	1.73	m	28.4
	2.26	m	
CH-2‴	5.43	m	126.2
C-3‴			135.3
CH$_2$-4‴	4.03	s	69.1
Me-C-3‴	1.66	s	13.8

[a] Recorded in $CDCl_3$. Chemical shift values are expressed as δ values (ppm) from TMS.
[b] s, singlet; d, doublet; dd, doublet of doublets; ddd, doublet of doublet of doublets; m, multiplet. J coupling values (Hz) are shown in parentheses.

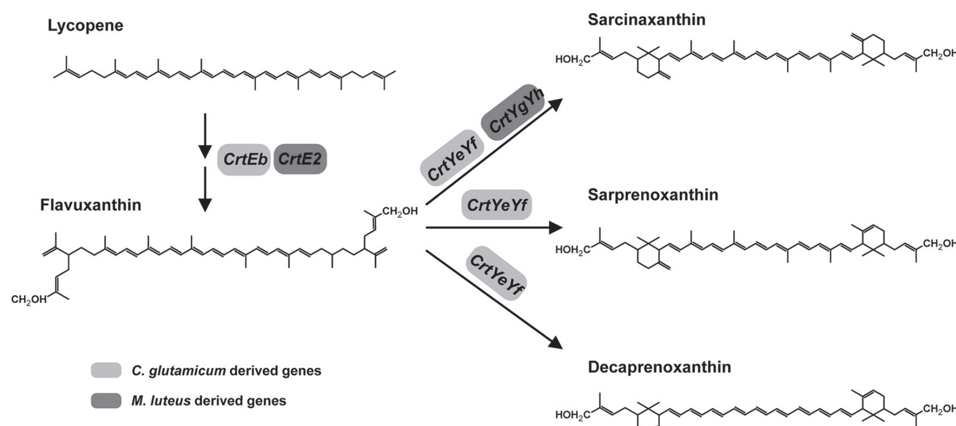

FIG. 6. Diverse biochemical functions of the *M. luteus* and the *C. glutamicum* C_{50} carotenoid cyclases. Biosynthesis of both sarcinaxanthin and decaprenoxanthin involves conversion of lycopene to flavuxanthin catalyzed by the lycopene elongases CrtEb and CrtE2 in *M. luteus* and *C. glutamicum*, respectively. The *M. luteus* CrtYgYh polypeptides constitute a γ-cyclase specifically converting flavuxanthin into sarcinaxanthin. In contrast, the *C. glutamicum* CrtYgYh polypeptides constitute both γ-cyclase and ε-cyclase activities and can convert flavuxanthin into three different C_{50} carotenoids; decaprenoxanthin, sarcinaxanthin, and sarprenoxanthin.

C. glutamicum. The reported presence of decaprenoxanthin glucosides in lycopene-producing *E. coli* cells expressing the *C. glutamicum crtEb*, *crtYe*, and *crtYf* genes led to the concomitant suggestion that host-encoded enzymes might be responsible for the glucosylation of decaprenoxanthin in *C. glutamicum* and *E. coli* (22). Based on their proximal localization and similar orientations, together with promoter prediction analyses, we propose that the sarcinaxanthin biosynthetic genes, including *crtX*, are cotranscribed from a common promoter located upstream of *crtE* in *M. luteus* (Fig. 2). The translational stop codons of *crtB*, *crtI*, *crtE2*, and *crtYg* overlap the translational start codons of their respective downstream genes, which may allow translational coupling to ensure equimolar expression and/or proper folding of the products. Secondary-structure analysis of the deduced gene products revealed six transmembrane helices for the *M. luteus* lycopene elongase CrtE2, and both CrtYg and CrtYh exhibited three transmembrane helices, indicating that they are transmembrane proteins (data not shown). This is in accordance with previous findings for carotenoid genes in other bacteria (4).

A new marine *M. luteus* isolate designated Otnes7 was included, together with the well-known *M. luteus* model strain NCTC2665. By expressing Otnes7 genes in a lycopene-producing *E. coli* host, we achieved complete conversion of the lycopene into production of 2.5 mg/g CDW sarcinaxanthin in shake flask cultures. Our data presented here indicate that Otnes7 genes may be favorable compared to *M. luteus* NCTC2665 genes when high-level heterologous production of sarcinaxanthin is desirable. Whether these differences are due to variations in the biochemical properties of the enzymes or, alternatively, different expression levels of the corresponding genes remains unknown. Our production experiments were done in small-scale shake flasks and without any optimization of growth conditions. We believe that conducting controlled high-cell-density cultivations of the recombinant *E. coli* strains under controlled conditions should further increase sarcinaxanthin production yields. However, this was not within the scope of the present study. In any case, our results implied that engineering *E. coli* host strains for higher lycopene production is presumably the immediate bottleneck for achieving increased sarcinaxanthin production.

The *M. luteus* CrtYg and CrtYh polypeptides were shown here to catalyze the *in vivo* γ-cyclization of the linear C_{50} carotenoid flavuxanthin to bicyclic sarcinaxanthin, and this cyclization reaction is independent of the prior stepwise lycopene elongation. In *C. glutamicum*, the C_{50} ε-cyclization reaction was reported by Krubasik et al. (22) to also be catalyzed by a heterodimeric protein encoded by *crtYe* and *crtYf*. However, in a separate report by these authors (24), different carotenoid intermediates were identified in *C. glutamicum* strains, and thus, an aberrant decaprenoxanthin pathway with a variant reaction sequence was proposed. Here, lycopene is transformed via monocyclic C_{45} nonaprene to bicyclic C_{50} sarcinene by two separate elongation/cyclization steps before decaprenoxanthin is formed by two sequential hydroxylation reactions. To our knowledge, the genetic basis for this alternative pathway is unknown. It should be noted that sarcinene has also been detected in *M. luteus* cell extracts (28), but whether this is relevant to sarcinaxanthin biosynthesis remains unclear. No sarcinene or nonaprene intermediates were detected in the present study. In *Dietzia* sp. CQ4, the conversion of the linear C_{50} carotenoid intermediate C.p.496 to the bicyclic C.p.450 has been reported to be catalyzed by a β-cyclase encoded by *lbtA* and the *lbtB* region in the *lbtBC* genes. The *lbtB*-encoded β-cyclase subunit appeared to be fused with a lycopene elongase (encoded by the *lbtC* region in the *lbtBC* genes) (41). The authors proposed a two-step sequential reaction consisting of the stepwise elongation of lycopene by the addition of two C_5 units and subsequent C_{50} β-cyclization, analogous to the reaction cascade in *M. luteus* proposed here. The *M. luteus* CrtYgYh enzyme represents the first

specific C_{50} carotenoid γ-cyclase that has been reported in the scientific literature.

Three different types of carotenoid cyclases are currently known, and based on sequence homology, the C_{50} carotenoid cyclases from *Dietzia* sp. and *C. glutamicum* have been proposed to be members of the CrtYcd-type cyclases (21). In contrast to other crtYcd-type cyclases, the C_{50} carotenoid cyclases are not involved in lycopene cyclization (41). Taking this together with our data presented here, we suggest that the C_{50} cyclases constitute a novel subtype of carotenoid cyclases conferring β-, ε-, and γ-cyclization of C_{50} carotenoids.

Carotenoid cyclases usually produce only one kind of ring structure (2), but lycopene cyclase from the cyanobacterium *Prochlorococcus marinus* has both β- and ε-cyclase activities (40). Surprisingly, construction and expression of a hybrid operon carrying the *M. luteus crtE2* gene and the C_{50} carotenoid ε-cyclase genes *crtYeYf* from *C. glutamicum* resulted in accumulation of three different C_{50} carotenoids in *E. coli*: ε-cyclic decaprenoxanthin, γ-cyclic sarcinaxanthin, and a new molecule containing both ε- and γ-rings, here designated sarprenoxanthin (Fig. 6). This result proved that the *C. glutamicum* cyclase encoded by *crtYeYf* can function both as an ε- and a γ-cyclase when expressed heterologously in *E. coli*, and thus, it has not one but multiple catalytic functions under the conditions tested. It was plausible to assume that the formation of sarprenoxanthin might be an artifact of overexpression; however, the same carotenoid products were detected in extracts of cells grown under noninduced conditions (data not shown). These results are contradictory to previous reports (22, 24), as neither sarcinaxanthin nor sarprenoxanthin has been identified in cell extracts of *C. glutamicum* strains or in cell extracts of recombinant *E. coli* strains expressing decaprenoxanthin biosynthetic genes.

In summary, we have unraveled the biosynthetic pathway of the γ-bicyclic C_{50} carotenoid sarcinaxanthin in *M. luteus*, including the function of the *crtX* gene product as sarcinaxanthin glycosyl transferase. In particular, our studies have generated new and important insight into the diverse catalytic functions of natural carotenoid cyclases as a novel class of enzymes directing biosynthesis of structurally different C_{50} carotenoids. Recently, the C_{40} carotenoid biosynthetic pathway of *Brevibacterium linens* was redesigned and extended by recruitment of heterologous genes leading to production of unexpected carotenoids in *E. coli* hosts (19). As we see it, C_{50} cyclases should represent interesting targets for future synthetic biology approaches aiming at generating structurally diverse carotenoids with interesting properties that might not be present in nature.

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