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Research Article

Heterologous carotenoid production in *Saccharomyces cerevisiae* induces the pleiotropic drug resistance stress response

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Abstract

To obtain insight into the genome-wide transcriptional response of heterologous carotenoid production in *Saccharomyces cerevisiae*, the transcriptome of two different *S. cerevisiae* strains overexpressing carotenogenic genes from the yeast *Xanthophyllomyces dendrorhous* grown in carbon-limited chemostat cultures was analysed. The strains exhibited different absolute carotenoid levels as well as different intermediate profiles. These discrepancies were further sustained by the difference of the transcriptional response exhibited by the two strains. Transcriptome analysis of the strain producing high carotenoid levels resulted in specific induction of genes involved in pleiotropic drug resistance (PDR). These genes encode ABC-type and major facilitator transporters which are reported to be involved in secretion of toxic compounds out of cells. β-Carotene was found to be secreted when sunflower oil was added to the medium of *S. cerevisiae* cells producing high levels of carotenoids, which was not observed when added to *X. dendrorhous* cells. Deletion of *pdr10*, one of the induced ABC transporters, decreased the transformation efficiency of a plasmid containing carotenogenic genes. The few transformants that were obtained had decreased growth rates and lower carotenoid production levels compared to a *pdr5* deletion and a reference strain transformed with the same genes. Our results suggest that production of high amounts of carotenoids in *S. cerevisiae* leads to membrane stress, in which Pdr10 might play an important role, and a cellular response to secrete carotenoids out of the cell. Copyright © 2010 John Wiley & Sons, Ltd.

Keywords: ***Saccharomyces cerevisiae*; heterologous carotenoid production; continuous culture; DNA microarray; pleiotropic drug resistance**

Introduction

Carotenoids are a class of pigments of commercial interest exerting important biological functions. In humans, β-carotene is the precursor for vitamin A; it may function as an anti-oxidant, has protective properties against cancer and stimulates the immune system (Palozza and Krinsky, 1992). Because carotenoids are coloured compounds, they are being utilized as pigments in the food and feed industries (Nelis and De Leenheer, 1991). Many carotenoids are being produced by chemical synthesis, which yield products that are pure and cheap (Gordon and Bauernfeind, 1982). Several microorganisms, including fungi, bacteria and algae, are able to produce carotenoids naturally

(Johnson and Schroeder, 1996). One example of a carotenoid-producing yeast is the red yeast *Xanthophyllomyces dendrorhous* (formerly known as *Phaffia rhodozyma*) (Golubev, 1995). This yeast mainly produces the carotenoid astaxanthin but also accumulates β-carotene as an intermediate of the astaxanthin biosynthesis pathway (Girard *et al.*, 1994). The genes involved in β-carotene production in *X. dendrorhous* have been cloned previously (Verdoes *et al.*, 1999a; Verdoes *et al.*, 1999b). In *X. dendrorhous*, the ergosterol and carotenoid biosynthetic pathways are connected by their utilization of prenyl pyrophosphates (Figure 1). Farnesyl pyrophosphate (FPP) is converted into geranylgeranyl pyrophosphate (GGPP) by GGPP synthase encoded by *crtE*. Next, phytoene synthase activity of the bifunctional enzyme CrtYB results in the synthesis of phytoene, using two GGPP molecules. Phytoene is subsequently converted into lycopene by four desaturation reactions catalysed by the enzyme CrtI. Subsequently, two cyclization reactions catalysed by CrtYB result in cyclization of lycopene to γ-carotene and finally β-carotene.

Previous work has shown that carotenoid production in *X. dendrorhous* can be increased by metabolic engineering (Visser *et al.*, 2003). However, *Saccharomyces cerevisiae* may be an attractive alternative, due to its well-known genetic and fermentation characteristics. We previously transformed *S. cerevisiae* with carotenogenic genes from *X. dendrorhous* (Verwaal *et al.*, 2007; Figure 1). Introduction of *crtYB* and *crtI* into *S. cerevisiae* resulted in a low level of carotenoid production. Additional introduction of geranylgeranyl pyrophosphate (GGPP) synthase from *S. cerevisiae* encoded by *BTS1* or GGPP synthase encoded by *crtE* from *X. dendrorhous* increases the carotenoid production levels in *S. cerevisiae*.

The genome-wide transcriptional response of *S. cerevisiae* cells engineered for carotenoid production might provide information concerning the impact on yeast physiology and identify targets for improvement for the production of these compounds. DNA microarray experiments have been shown to be a powerful tool to study the genome-wide transcriptional response of *S. cerevisiae* to changes of the physiological state and the environment (Bammert and Fostel, 2000; DeRisi *et al.*, 2000; Gasch *et al.*, 2000; Tai *et al.*, 2005). Genomics approaches on *S. cerevisiae* cells producing heterologous metabolites to study their impact on yeast physiology have been reported recently (Ro *et al.*, 2008). Most transcriptome studies with *S. cerevisiae* have been performed with cells grown in shake-flask cultures. The main drawback of shake-flask cultivation is that the environment is continuously changing, which may have a big influence on carotenoid production and interpretation of transcriptome data (Regenberg *et al.*, 2006). Chemostat cultivation offers advantages for studies with DNA microarrays because it enables cultivation of microorganisms under tightly defined environmental conditions (Daran-Lapujade *et al.*, 2009). An interlaboratory comparison of transcriptome data obtained in chemostat cultures has indeed demonstrated that the accuracy and reproducibility of this

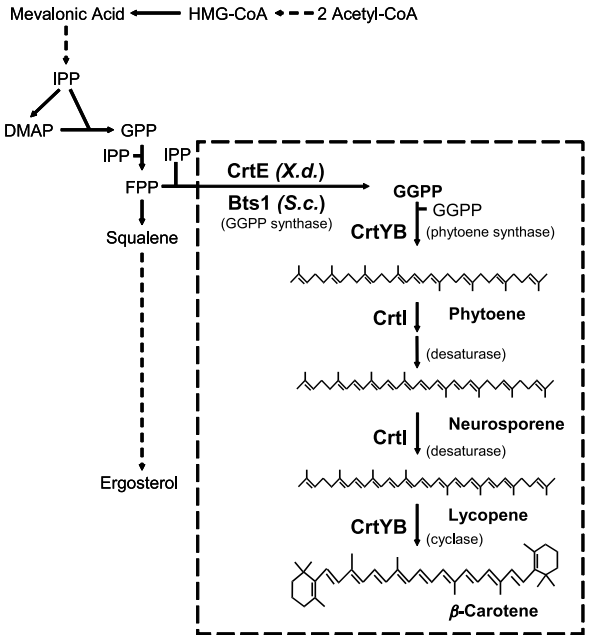

Figure 1. β-carotene formation in *X. dendrorhous* and *S. cerevisiae*. The carotenogenic pathway of *X. dendrorhous* was introduced into *S. cerevisiae* (boxed area). It consists of GGPP synthase, encoded by *crtE*, the bifunctional enzyme phytoene synthase and lycopene cyclase encoded by *crtYB* and phytoene desaturase encoded by *crtI*. *S. cerevisiae* expresses an endogenous GGPP synthase, encoded by *BTS1*, which is able to convert FPP into GGPP. The ergosterol biosynthetic pathway, in both *S. cerevisiae* and *X. dendrorhous*, provides precursors for carotenoids. Solid arrow, direct conversion; dashed arrow, indirect conversion; IPP, isopentenyl pyrophosphate; DMAP, dimethylallyl pyrophosphate; GPP, geranyl pyrophosphate; FPP, farnesyl pyrophosphate; GGPP, geranylgeranyl pyrophosphate

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approach are superior to those obtained in previous studies with shake-flask cultures (Piper *et al*., 2002).

In this study, chemostat cultivation and transcriptome analysis were combined to study the effect of heterologous carotenoid production in *S. cerevisiae* on the physiology and the transcriptional response. For this purpose, two strains producing low and high levels of carotenoids were grown in aerobic glucose-limited (C-limited) chemostat cultures and analysed using Affymetrix GeneChip® DNA microarrays. High carotenoid production levels resulted in induced expression of genes involved in pleiotropic drug resistance (PDR), including multidrug resistance and major facilitator transporters, which are implicated in various pleiotropic reactions, such as membrane stress response and transport of a large number of compounds (Jungwirth and Kuchler, 2006). Our results suggest that high carotenoid production levels lead to membrane stress and that by activation of multidrug resistance transporters the produced carotenoids are directed towards the extracellular space.

Materials and methods

Strains

The strains used in this study are listed in Table 1. Strain Orange01 was constructed by genomic integration of YIplac211 containing *crtYB, crtI* (from *X. dendrorhous)* and *BTS1* (from *S. cerevisiae*). Strain Orange02 was constructed by genomic integration of YIplac211 containing *crtYB, crtI* and *crtE* (all from *X. dendrorhous*). Expression of all genes was controlled by the *TDH3* promoter and *CYC1* terminator. Detailed construction of the expression plasmids and strains was described previously (Verwaal *et al*., 2007). The *Xanthophyllomyces dendrorhous* reference strain CBS6938 and the β-carotene accumulation mutant

Table 1. List of strains used in this study and relevant genotypes

Strain	Relevant features	Reference
CEN.PK 113-7D	*MAT***a**, *SUC2, MAL2-8*[c]	P. Kötter[a]
CEN.PK 113-5D	*MAT***a**, *SUC2, MAL2-8*[c], *ura3-52*	P. Kötter[a]
Orange 01	CEN.PK 113-5D *ura3-52*::P*TDH3-crtYB*-T*CYC1*; P*TDH3-crtI*-T*CYC1*; P*TDH3-BTS1*-T*CYC1* (*URA3*)	(Verwaal *et al*., 2007)
Orange 02	CEN.PK 113-5D *ura3-52*::P*TDH3-crtYB*-T*CYC1*; P*TDH3-crtI*-T*CYC1*; P*TDH3-crtE*-T*CYC1* (*URA3*)	(Verwaal *et al*., 2007)
BY4741 and deletion strains	*MAT***a**, *his3Δ1, leu2Δ0, met15Δ0, ura3Δ0*	EUROSCARF[b]
BY4741 YEplac195 or YB/I/E	BY4741 (Y00000) +YEplac195 or YEplac195 P*TDH3-crtYB*-T*CYC1*; P*TDH3-crtI*-T*CYC1*; P*TDH3-crtE*-T*CYC1*	This study
BY4741Δ*pdr5* YEplac195 or YB/I/E	BY4741 *YOR153w::kanMX4* (Y02409) + YEplac195 or YEplac195 P*TDH3-crtYB*-T*CYC1*; P*TDH3-crtI*-T*CYC1*; P*TDH3-crtE*-T*CYC1*	This study
BY4741Δ*pdr10* YEplac195 or YB/I/E	BY4741 *YOR328w::kanMX4* (Y01625) + YEplac195 or YEplac195 P*TDH3-crtYB*-T*CYC1*; P*TDH3-crtI*-T*CYC1*; P*TDH3-crtE*-T*CYC1*	This study
BY4741Δ*pdr15* YEplac195 or YB/I/E	BY4741 *YDR406W::kanMX4* (Y04242) + YEplac195 or YEplac195 P*TDH3-crtYB*-T*CYC1*; P*TDH3-crtI*-T*CYC1*; P*TDH3-crtE*-T*CYC1*	This study
BY4741Δ*snq2* YEplac195 or YB/I/E	BY4741 *YDR011w::kanMX4* (Y03951) + YEplac195 or YEplac195 P*TDH3-crtYB*-T*CYC1*; P*TDH3-crtI*-T*CYC1*; P*TDH3-crtE*-T*CYC1*	This study
BY4741Δ*flr1* YEplac195 or YB/I/E	BY4741 *YBR008c::kanMX4* (Y03143) + YEplac195 or YEplac195 P*TDH3-crtYB*-T*CYC1*; P*TDH3-crtI*-T*CYC1*; P*TDH3-crtE*-T*CYC1*	This study
BY4741Δ*tpo1* YEplac195 or YB/I/E	BY4741 *YLL028w::kanMX4* (Y01516) + YEplac195 or YEplac195 P*TDH3-crtYB*-T*CYC1*; P*TDH3-crtI*-T*CYC1*; P*TDH3-crtE*-T*CYC1*	This study
CBS6938	*Xanthophyllomyces dendrorhous* reference	*CBS*[c]
PR-1-104	β-Carotene accumulation mutant of *X. dendrorhous*	(Girard *et al*., 1994)

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[b] EUROSCARF (**http://web.uni-frankfurt.de/fb15/mikro/euroscarf/**). EUROSCARF accession numbers are mentioned between brackets.
[c] Centraal Bureau Schimmelcultures (**http://www.cbs.knaw.nl/**).

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PR-1-104 (Girard *et al.*, 1994) were also used in this study. Single *S. cerevisiae* deletion mutants were obtained from the BY4741 MAT**a** deletion collection from EUROSCARF (**http://web.uni-frankfurt.de/fb15/mikro/euroscarf/**) (Table 1) and were transformed with YEPlac195 YB/I/E (Verwaal *et al.*, 2007) and YEPlac195 as control. Transformation plates were incubated for 3 days at 30 °C.

Strain cultivations

For chemostat cultivation, strains CEN.PK113-7D, Orange01 and Orange02 were grown at 30 °C in 2 litre chemostats (Applikon) with a working volume of 1.0 l, as described (van den Berg *et al.*, 1996). Cultures were fed with a defined synthetic medium under glucose limitation (C-limited) with all other growth requirements in excess and at a constant residual concentration. Strains CEN.PK113-7D and Orange01 were grown on synthetic medium (Verduyn *et al.*, 1990), containing 7.5 g/l glucose to maintain carbon limitation. The synthetic medium contains the following components: $(NH_4)_2SO_4$, 5 g/l; KH_2PO_4, 3 g/l; $MgS0_4.7H_2O$, 0.5 g/l; EDTA, 15 mg/l; $ZnSO_4.7H_2O$, 4.5 mg/l; $CoC1_2.6H_2O$, 0.3 mg/l; $MnC1_2.4H_2O$, 1 mg/l; $CuSO_4.5H_2O$, 0.3 mg/l; $CaC1_2.2H_2O$, 4.5 mg/l; $FeSO_4.7H_2O$, 3 mg/l; $NaMoO_4.2H_2O$, 0.4 mg/l; H_3BO_3, 1 mg/l; KI, 0.1 mg/l; and 0.025 ml silicone antifoam (BDH). Filter-sterilized vitamins were added after heat sterilization (120 °C) of this medium. Final vitamin concentrations: biotin, 0.05 mg/l; calcium pantothenate, 1 mg/l; nicotinic acid, 1 mg/l; inositol, 25 mg/l; thiamine HCl, 1 mg/l; pyridoxine HCl, 1 mg/l; and *p*-aminobenzoic acid, 0.2 mg/l. Strain Orange02 was grown on synthetic medium, containing 7.5 g/l glucose supplemented with 9.38 mM ethanol to avoid metabolic oscillations (Cipollina *et al.*, 2008). To compare strain Orange02 to reference cells, strain CEN.PK113-7D was also grown on this medium. The dilution rate was set at 0.10/h. The pH was measured online and kept constant at 5.0 by the automatic addition of 2 M KOH, using an Applikon ADI 1030 biocontroller. The stirrer speed was 800 rpm and the airflow was 0.5 l/min. Dissolved oxygen tension was measured online with an Ingold model 34-100-3002 probe and was above 50% of air saturation. The off-gas was cooled by a condenser connected to a cryostat set at 2 °C, and oxygen and carbon dioxide were measured off-line with an NGA 2000 Rosemount gas analyser. Steady-state samples were taken after ~10–14 volume changes to avoid strain adaptation due to long-term cultivation (Ferea *et al.*, 1999; Jansen *et al.*, 2005). Dry weight, glucose and ethanol levels, dissolved oxygen and gas profiles had to be constant over at least five volume changes before sampling for RNA extraction.

Shake-flask cultivation for the determination of intracellular accumulation of total carotenoids were performed as follows: Cells were inoculated in 500 ml flasks containing 100 ml defined synthetic medium (5 g/l $(NH_4)_2SO_4$, 3 g/l KH_2PO_4, 0.5 g/l $MgSO_4$ and trace elements) supplemented with 20 g/l glucose, vitamins and auxotrophic requirements histidine, leucine, methionine and uracil, as previously described (Pronk, 2002) with an initial pH of 6.0. The flasks were incubated at 30 °C on an orbital shaker set at 200 rpm. Growth of different strains was monitored by optical density (OD) measurements at 660 nm.

Analytical methods

Culture supernatants were obtained after centrifugation of samples from the chemostats. For the purpose of glucose determination and carbon recovery, glucose, ethanol, glycerol, acetate and pyruvate concentrations in the supernatants of chemostat cultures were determined by high-performance liquid chromatography (HPLC) analysis, using an HPX-87H Aminex ion-exchange column (300 × 7.8 mm; Bio-Rad) at 60 °C. The column was eluted with 5 mM sulphuric acid at a flow rate of 0.6 ml/min. Pyruvate and acetate were detected at 214 nm with a Waters 441 UV meter coupled to a Waters 741 data module. Glucose, ethanol and glycerol were detected with an ERMA type ERC-7515A refractive index detector coupled to a Hewlett-Packard type 3390A integrator. Culture dry weights were determined via filtration as described (Postma *et al.*, 1989).

Carotenoid secretion was determined as follows. *S. cerevisiae* strains (CEN.PK113-7D and Orange02) and *X. dendrorhous* strains (CBS6938 and PR-1-104) were grown in 20 ml YPD (5 g/l Bacto-yeast extract, 10 g/l Bacto peptone and 2% v/v D-glucose) in 250 ml shake flasks. Sunflower oil (Fluka) was added to the growth medium to a final concentration of 20% v/v. After 72 h

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of growth at 30 °C on an orbital shaker set at 225 rpm, the cultures were centrifuged for 10 min at 4000 rpm. Carotenoids were extracted from the oil phase as follows: 3 ml oil sample was mixed with 20 ml methanol containing 10% KOH and heated for 30 min at 60 °C in an Erlenmeyer flask. The air was removed by a stream of nitrogen; the flask was sealed and kept in the dark for 18 h. Then the carotenoids were partitioned into 10% ether in petrol and the composition was determined by HPLC (Sandmann, 2002).

Intracellular accumulation of different types of carotenoids was determined by HPLC as described previously (Verwaal *et al*., 2007). Intracellular accumulation of total carotenoids was analysed by resuspension of the cells in 1 ml sterile water, 1 g 0.50–0.75 mm glass beads was added and the cells were broken by vortexing for 3 min. 2.5 ml 0.2% w/v pyrogallol dissolved in methanol was added and the cells were vortexed for 10 s. After adding 1.25 ml 60% w/v KOH and vortexing for 10 s, the cells were incubated for 1 h at 75 °C with vortexing every 15 min for saponification. Next, an appropriate amount of hexane was added to extract the carotenoids. The tubes were centrifuged for 5 min at 2800 rpm and 1 ml of the hexane layer was pipetted into a cuvette. Absorption between 550 and 400 nm was monitored on a Shimadzu UV-2501 PC spectrophotometer (Shimadzu, Duisburg, Germany). A standard curve was determined by measuring known β-carotene concentrations. The total carotenoid concentration was calculated by the following formula, which was derived from a standard curve:

$$\text{Total carotenoids (in } \mu\text{g/g dw)} = (A_{449\ \text{nm}} \times \text{ml hexane})/(0.2072 \times \text{g dw}).$$

The dry weight (dw) of a sample was measured by taking the same culture volume as used for carotenoid extraction, drying overnight at 80 °C and measuring the weight of the dried yeast cells on an analytical balance.

Microarray analysis

Sampling of the Orange01 strain cells and its isogenic reference CEN.PK113-7D from aerobic C-limited chemostats with glucose as sole carbon source, probe preparation and hybridization to Affymetrix GeneChip® microarrays were performed as described previously (Piper *et al*., 2002). Sampling of strains Orange02 and its isogenic reference CEN.PK113-7D from aerobic C-limited chemostats with glucose/ethanol mixture, probe preparation and hybridization to Affymetrix GeneChip microarrays were performed as described previously (Piper *et al*., 2002) with the following modifications: double-stranded cDNA synthesis was carried out using 15 µg total RNA and the components of the One Cycle cDNA Synthesis Kit (Affymetrix). Double-stranded cDNA was purified (GeneChip Sample Cleanup Module, Qiagen) before *in vitro* transcription and labelling (GeneChip IVT Labelling Kit, Affymetrix). Finally, labelled cRNA was purified (GeneChip Sample Cleanup Module) prior to fragmentation and hybridization of 15 µg biotinylated cRNA. The results for each growth condition were derived from three or more independently cultured replicates (De Nicola *et al*., 2007).

Data acquisition and analysis

Acquisition and quantification of array images and data filtering were performed using Affymetrix Microarray Suite v 5.0, MicroDB v 3.0, and Data Mining Tool v 3.0. Before comparison, all arrays were globally scaled to a target value of 150, using the average signal from all gene features using Microarray Suite Version 5.0. To eliminate insignificant variations, genes with values below 12 were set to 12 as described (Piper *et al*., 2002). From the 9335 transcript features on the YG-S98 arrays, a filter was applied to extract 6383 yeast open reading frames, representing 6084 different genes. The discrepancy was due to several genes being present more than once, as suboptimal probe sets were used in the array design. To account for variation in the triplicate measurements, the coefficient of variation (SD divided by the mean) was calculated as described previously (Boer *et al*., 2003). For additional statistical analyses, Microsoft Excel running the significance analysis of microarrays (SAM Version 1.12) add-in was used for pairwise comparisons (Tusher *et al*., 2001). Genes were considered as being changed in expression if they were called significantly changed by at least two-fold from each other using SAM (expected median false discovery rate of 1%). The microarray data have been deposited at Genome Expression Omnibus database (**http://www.ncbi.nlm.nih.gov/geo/**) under the

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Table 2. List of primers used for qPCR

Gene	Forward primer	Reverse primer
Kanamycin	AGCATTACGCTGACTTGACG	AGGTCGACCAGTTGGTGATT
PDR5	GTGGTGTTATGACTACCCCAAGT	CATGTACTGCCCACATGTCATA
PDR10	TGCTCTGCTATCGGTAGGACT	CTCCCATCGAGCAGATAACC
PDR15	ACTCCACCATCTGGAACCAC	AAAGGCATTGGTGGTGGATA
SNQ2	CACAACCTGTCTCATTGATGC	GGTTTCATGTACTCTCCACACG
TPO1	CATGAGGTCTGCATTTGGTG	GTAAAGGCACGGGAATCATC
FLR1	GGCTACATGCTACCCAAAGTATG	GGAACCCCACTAAGGACGA
OYE3	GGAAGGGTCCAATCATCAGA	CGGACATGGTGTAGAAGGT
FET3	GCCATGACATTCTCCTGCTT	TGCTTTTCAGTGGAATGACG
SPS100	CACAACAGTACCGTGGCATC	TGGTGACAAGTACCCAGCAG

All sequences are depicted from 5′ to 3′.

Series No. GSE8451. Promoter analysis was performed using the web-based software Regulatory Sequence Analysis (RSA) tools (van Helden *et al*., 1998). The statistical assessment of overrepresentation of GO biological processes categories (**http://www.geneontology.org/**) among sets of significantly changed transcripts was achieved as previously described in (Kresnowati *et al*., 2006). Heat-map visualizations of the average normalized transcript data were generated with Expressionist Analyst v 3.2 (Genedata, Basel, Switzerland).

Quantitative PCR (qPCR) analysis

Total RNA was isolated from CEN.PK113-7D and Orange02 as described under Microarray analysis, because the same samples used for the DNA microarray experiments were used for qPCR. cDNA amplification, primer design and qPCR experiments were performed as described previously (Verwaal *et al*., 2007). As internal control for cDNA amplification, 1 ng 1.2 kb kanamycin-positive control RNA (Promega, Leiden, The Netherlands) was added to 1 μg total RNA. Primers used for qPCR are indicated in Table 2. The results are presented as ratios of gene expression between the target gene (gene of interest) and the reference kanamycin (Pfaffl, 2001).

Results

Physiology and carotenoid production during growth in glucose-limited chemostat cultures

The yeast *S. cerevisiae* was transformed into a carotenoid-producing organism by introduction and overexpression of carotenogenic genes from the yeast *X. dendrorhous* to create the recombinant strains Orange01 and Orange02 (Verwaal *et al*., 2007). Strains Orange01 (low carotenoid production levels) and Orange02 (high carotenoid production levels) were grown in aerobic C-limited chemostat cultures at a dilution rate of 0.1/h. However, growth of strain Orange02 was disturbed by metabolic oscillations during growth on synthetic medium, which resulted in a 3.5 h periodical change in the dissolved oxygen content. Addition of 9.38 mM ethanol to the culture medium prevented oscillation during growth of strain Orange02 (data not shown), as observed previously (Cipollina *et al*., 2008). In order to correctly compare strain Orange02 to the isogenic reference, strain CEN.PK113-7D was also grown in aerobic C-limited chemostat cultures in the presence of 9.38 mM ethanol. For each culture, the biomass yield, the glucose and oxygen consumption rates and the ethanol and carbon dioxide production were determined (Table 3). The physiological parameters of cells producing carotenoids and the reference strain grown under the same conditions were comparable. Only the biomass of carotenoid-producing cells was slightly lower compared to control cells. The yield of cells grown in the presence of a low amount of ethanol in the growth medium was slightly higher, probably because ethanol was used for biomass production. The biomass yield was very close to 0.5 g/g, indicating complete respiratory catabolism, which is characteristic of steady-state growth of *S. cerevisiae* strain CEN.PK113-7D under glucose limitation at dilution rates lower than 0.3/h (Diderich *et al*., 2002). With exception of the oscillatory behaviour, the constancy of the recorded parameters suggests

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Table 3. Physiological parameters of the cultures grown in this study

Strain	+ Ethanol	Y_{sx}[a]	$q_{glucose}$[b]	$q_{ethanol}$[c]	qO_2[d]	qCO_2[e]	RQ[f]	CR[g]
CENPK113-7D (ref. 1)	No	0.49 ± 0.01	1.1 ± 0.0	0.0 ± 0.0	2.8 ± 0.3	2.8 ± 0.3	1.0 ± 0.0	98 ± 3
Orange01	No	0.47 ± 0.01	1.2 ± 0.0	0.0 ± 0.0	3.0 ± 0.1	2.8 ± 0.1	0.94 ± 0.02	97 ± 3
CENPK113-7D (ref. 1)	Yes	0.51 ± 0.01	1.1 ± 0.0	−0.2 ± 0.0	3.0 ± 0.1	2.9 ± 0.1	0.95 ± 0.02	100 ± 3
Orange02	Yes	0.50 ± 0.01	1.1 ± 0.0	−0.1 ± 0.0	3.0 ± 0.1	2.9 ± 0.0	0.96 ± 0.01	99 ± 4

All data represent the average and SD of three independent steady states, except those for isogenic reference CEN.PK113-7D grown in the presence of ethanol, which represent the average and SD of four independent steady states. The amount of ethanol added to the culture medium as indicated was 9.38 mM.

[a] Yield of biomass (g/g glucose consumed).

[b] mM glucose consumed/g biomass/h.

[c] mM ethanol produced/g biomass/h.

[d] mM oxygen consumed/g biomass/h.

[e] mM carbon dioxide produced/g biomass/h.

[f] RQ, respiratory quotient (qCO_2/qO_2).

[g] CR, carbon recovery.

Table 4. Intracellular accumulation of different carotenoids in isogenic reference strain CEN.PK113-7D, strain Orange01 and strain Orange02 during growth in batch and continuous cultures, as determined by HPLC

	Specific amount ± SD [$\mu g/g_{dry\ weight}$ (% distribution) of carotenoids]					
Carotenoid	**CEN.PK113-7D (batch)**	**CEN.PK113-7D (Chemostat)**	**Orange01 (batch)**	**Orange01 (Chemostat)**	**Orange02 (batch)**	**Orange02 (Chemostat)**
Phytoene	ND	ND	476 ± 88 (94)	13 ± 6 (6)	1323 ± 66 (86)	1578 ± 481 (91)
Neurosporene	ND	ND	3 ± 1 (0.5)	11 ± 2 (5)	5 ± 1 (0.5)	ND
Lycopene	ND	ND	ND	ND	ND	111 ± 43 (6)
β-Zeacarotene	ND	ND	4 ± 1 (0.5)	ND	16 ± 3 (1)	ND
7,8-Dihydro-β-carotene	ND	ND	10 ± 2 (2)	ND	60 ± 1 (3.5)	ND
γ-Carotene	ND	ND	10 ± 2 (2)	ND	ND	23 ± 11 (1)
β-Carotene	ND	ND	15 ± 3 (3)	187 ± 38 (89)	141 ± 5 (9)	41 ± 23 (2)
Total	0	0	507 ± 82	211 ± 34	1545 ± 65	1752 ± 531

The reported data represent the average ± SD of two independent batch cultures and three independent chemostat cultures. The relative levels of individual carotenoids are indicated as percentages of totally produced carotenoids.
ND, not detected.

that carotenoid production does not affect yeast physiology.

At steady state, strain Orange01 displayed a yellow colour during growth in the fermentor, whereas the colour of strain Orange02 was much more orange (data not shown). To determine which carotenoids accumulated in the recombinant strains grown in C-limited continuous cultures, samples taken at steady state were analysed by HPLC and compared with carotenoid production data obtained from batch cultures (Table 4). No carotenoids accumulated in the reference strain during growth in batch and chemostat cultures. Strain Orange01 produced lower total carotenoid levels in chemostat cultures compared to the batch cultures. Furthermore, the results showed that the carotenoid pools were very different between the two culture formats: in batch cultures a clear accumulation of phytoene (up to 94%) was observed, while in the chemostat cultures mainly β-carotene accumulated (89% of total carotenoids). On the other hand, in chemostat cultures, strain Orange02 produced similar total carotenoid levels compared to growth in batch cultures (Table 4). In contrast to strain Orange01, the accumulated carotenoids were comparable in both culture systems, with a clear enrichment in phytoene (up to 91% of total carotenoids, Table 4). Despite the lower β-carotene concentration in strain Orange02 compared to strain Orange01, it exhibited a more orange colour, which is probably caused by the higher orange γ-carotene and reddish lycopene

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content. To determine the effect of heterologous carotenoid production on the transcriptome, samples of the two carotenoid-producing strains grown in aerobic C-limited chemostat cultures were further processed to perform DNA microarray experiments.

Global transcriptome analysis

Genome-wide transcriptome analysis was performed on cells grown in aerobic C-limited chemostat cultures, producing low (Orange01, 211 μg/$g_{dry\ weight}$) or high (Orange02, 1752 μg/$g_{dry\ weight}$) total carotenoids amounts. To analyse the effects of heterologous carotenoid production on gene expression, DNA microarrays derived from carotenoid-producing cells were compared to those of the isogenic reference strain CEN.PK113-7D, grown under similar conditions (presence of 9.38 mM ethanol for strain Orange02 or absence of ethanol for strain Orange01). Each strain was cultured at least in triplicate (Piper *et al.*, 2002). In terms of reproducibility, the average coefficient of variation for the transcriptome analyses for each of the four growth conditions was below 0.19. In addition, the level of the *ACT1* transcript, a common loading standard for conventional Northern analysis, varied less than 13% over the four growth conditions.

In strain Orange01, 47 genes were 1.5-fold or more induced and four genes were 1.5-fold or more repressed, whereas in strain Orange02, 36 genes were 1.5-fold or more induced, and 57 genes were 1.5-fold or more repressed (Figure 2). However, the overlap between the two datasets was very limited. Only five genes were upregulated in both strain Orange01 and strain Orange02 (*PDR5, PHO5, URA1, URA3* and *VHR1*). Induction of *URA3* and *URA1*, encoding proteins involved in biosynthesis of pyrimidines, is probably a consequence of the strain construction strategy, since the carotenogenic biosynthetic was inserted at the *ura3,52* locus of strain CEN.PK113-5D. Subsequently, the increase in *URA1* expression might be related to that of *URA3*. Of the remaining three genes, *PHO5* encodes a secreted acid phosphatase, which is involved in nucleotide-derived phosphate hydrolysis in the periplasmic space; *VHR1* encodes a transcriptional activator, required for the induction of vitamin H and biotin intermediate transporters *VHT1* and *BIO5*, respectively (Weider *et al.*, 2006); and *PDR5* encodes the plasma membrane ABC-transporter involved in cellular detoxification (Balzi *et al.*, 1994; Mahe *et al.*, 1996).

Fischer's exact test (Knijnenburg *et al.*, 2007) was applied to the sets of up- and downregulated genes obtained by comparing the Orange01 and Orange02 strains to their respective reference to assess enrichment for functional categories from the publicly available ontology database MIPS (Mewes *et al.*, 2004) (Table 5). Despite the number of genes (47) upregulated in the Orange01 strain, no significant ($p < 1.0E-04$) functional enrichment was detected. Similarly, no overrepresentation of genes with similar function was found in genes downregulated in the Orange01 strain. Analysis of the 36 genes upregulated in Orange02 revealed overrepresentation of MIPS categories related to detoxification and transport functions, comprising a total of eight genes (*FLR1, GRE2, PDR3, PDR5, PDR10, PDR15, SNQ2* and *TPO1*). In the meantime, genes with a lower expression level in the Orange02 strain exhibited enrichment in genes belonging to the 'cell rescue, defence and virulence' functional category. The large majority of this group is composed of genes encoding members of the seripauperin multigene family. The proteins encoded by these genes have yet uncharacterized functions; they possess transmembrane regions and are likely to be present at the surface of the cell.

To confirm some of the results from the DNA microarray studies, a qPCR experiment was performed. Expression of the genes *PDR5, PDR10, PDR15, SNQ2, FLR1, TPO1* and *OYE3* was induced and expression of *FET3* and *SPS100* was repressed in strain Orange02 as compared to the control strain CEN.PK113-7D (Figure 3).

Carotenoid production induces expression of genes involved in pleiotropic drug resistance

In-depth 5′-cis-regulatory sequence search analysis in the promoter of the upregulated genes in Orange02 revealed the presence of a canonical pleiotropic drug response element (PDRE) regulatory site (TCCGCGGA) in one-third of the promoter sequences, a known target of the transcription activator Pdr3. In *S. cerevisiae*, pleiotropic drug resistance is not solely mediated by Pdr3 but may involve other regulators, viz. Pdr1, Pdr8, Yrr1 and its paralogue Yrm1 (DeRisi *et al.*, 2000; Le Crom *et al.*, 2002; Hikkel *et al.*, 2003; Onda *et al.*, 2004; Lucau-Danila *et al.*, 2005). This response

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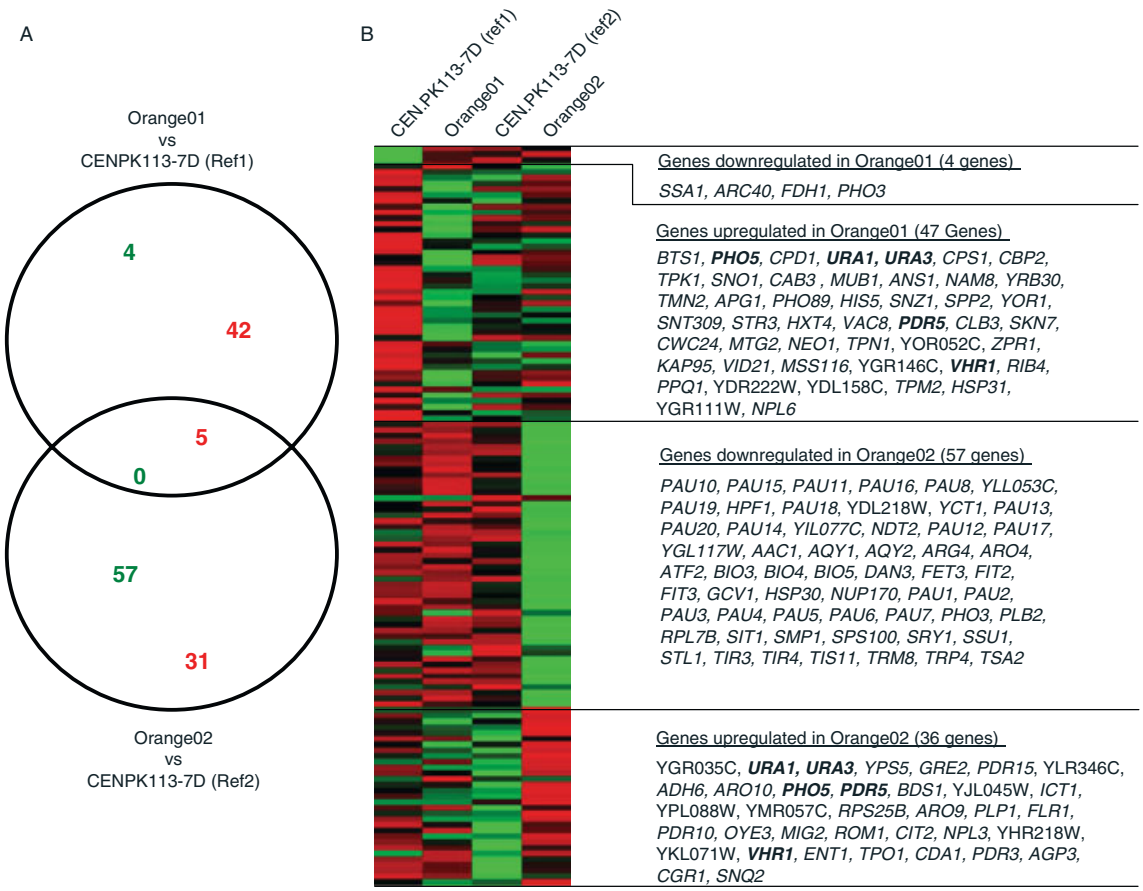

Figure 2. Induced and repressed genes in strain Orange01 and Orange02 during growth in aerobic C-limited chemostat cultures. (A) Venn diagram. (B) The average of three replicate transcription profiles of strain Orange01 and six replicate transcription profiles of strain CEN.PK113-7D (reference 1) were averaged and compared. For strain Orange02, the average of three replicate transcription profiles and four replicate transcription profiles of strain CEN.PK113-7D (reference 2) were averaged and compared. To strain Orange02 and CEN.PK113-7D (reference 2) 9.38 mM ethanol was added to prevent metabolic oscillations. The genes indicated in bold correspond to those upregulated in Orange01 and Orange02 relative to reference 1 and reference 2, respectively

Table 5. Global transcriptome analysis of strain Orange02

MIPS category	*k*	*n*	*p*	Genes
Orange2 vs CEN.PK113-7D *Upregulated genes*				
Metabolism of phenylalanine	3	16	9.5E-05	*ARO9, ARO10,* YPL088W
Drug/toxin transport	5	40	2.8E-06	*FLR1, PDR5, PDR10, SNQ2, TPO1*
ABC transporters	4	28	1.8E-05	*PDR5, PDR10, PDR15, SNQ2*
Disease, virulence and defence	4	33	3.5E-05	*FLR1, PDR3, PDR5, SNQ2*
Detoxification	6	119	5.4E-05	*FLR1, GRE2, PDR3, PDR5, SNQ2, TPO1*
Downregulated genes				
Cell rescue, defence and virulence	27	559	3.4E-06	*FET3, HSP30, PAU1, PAU2, PAU3, PAU4, PAU5, PAU6, PAU7, PAU8, PAU9, PAU10, PAU11, PAU12, PAU13, PAU14, PAU15, PAU16, PAU17, PAU18, PAU19, PAU20, SIT1, SSU1, TIR3, TIR4, TSA2*

Identification using Fischer exact test of enriched (p < 01.0E-04) biological themes of genes showing a significant change in expression in the high β-carotene-producing strain Orange02 compared to its isogenic reference CEN.PK113-7D.
k, number of genes in the differentially expressed set; *n*, number of genes in a certain MIPS category (Mewes *et al*., 2004).

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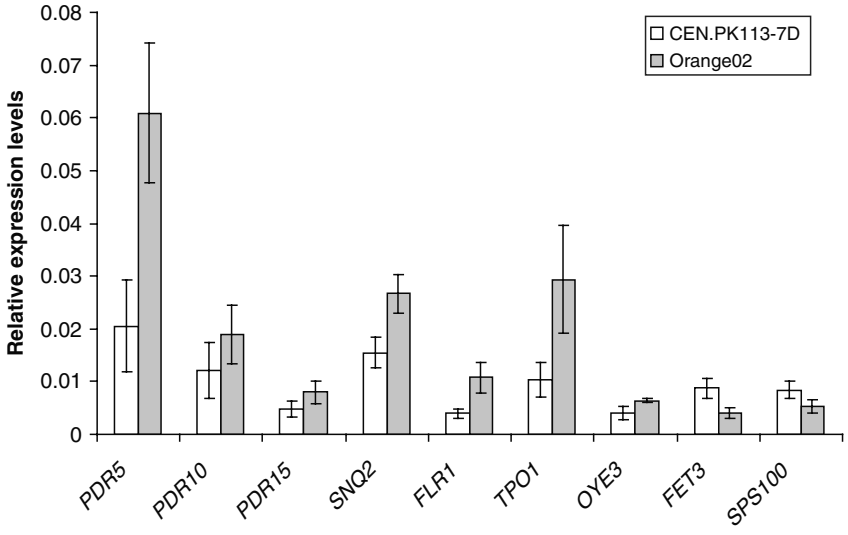

Figure 3. Induced expression of multidrug resistance transporter in strain Orange02 during growth in C-limited continuous cultures, as determined by qPCR experiments. Relative expression levels of *PDR5, PDR10, PDR15, SNQ2, FLR1, TPO1, OYE3, FET3* and *SPS100* in strain Orange02 and reference strain CEN.PK113-7D were determined. Averages and standard deviations of four individual CEN.PK113-7D cultures and three individual Orange02 cultures are indicated. Kanamycin was used as the internal standard for normalization

includes increased exocytosis, decreased intracellular pH, cellular detoxification, changes in membrane composition and overexpression of drug efflux pumps (Jungwirth and Kuchler, 2006). Using YEASTRACT, a web-based transcription factor database (Teixeira *et al.*, 2006), we determined which of the 36 induced genes are known to be regulated by the transcription factors Pdr1, Pdr3, Pdr8, Yrr1 or Yrm1. Subsequently, 16 of the 36 induced genes were identified as being regulated by one or more of the transcription factors Pdr1, Pdr3, Pdr8, Yrr1 or Yrm1 (Table 6). This indicated that expression of a highly significant percentage of induced genes was controlled by transcription factors involved in regulation of pleiotropic drug resistance.

Of the 16 genes that have previously been shown to be controlled by one or more of the five transcription factors, we counted six transporters, of which four were members of the ATP-binding cassette (ABC) transporter, *PDR5* (Balzi *et al.*, 1994), *PDR10* (Wolfger *et al.*, 1997), *PDR15* (Wolfger *et al.*, 2004) *SNQ2* (Servos *et al.*, 1993), and the remaining two were member of the major facilitator superfamilies *TPO1* (Tomitori *et al.*, 1999) and *FLR1* (Alarco *et al.*, 1997). The 11 remaining genes that were identified in this search do not encode multidrug resistance transporters but have other or still unknown functions. The observed induction of four ABC transporters and two major facilitator transporters suggests that these transporters might be involved in secretion of carotenoids out of the recombinant yeast cells. The induced PDR response also implies that heterologously produced carotenoids might be considered as toxic compounds for *S. cerevisiae* cells.

Carotenoid secretion from carotenoid-producing *S. cerevisiae* cells

If multidrug resistance transporters are involved in the secretion of carotenoids, one would expect that the produced carotenoids are secreted into the growth medium. However, growth of strain Orange02 did not result in colouring of the growth medium in batch cultures or during growth in chemostat cultures (data not shown). We reasoned that carotenoids, which are hydrophobic compounds (Gruszecki and Strzalka, 2005), cannot readily dissolve in the media used for the growth of yeast strains but are more likely to remain attached to cellular membranes. To determine whether carotenoids could be secreted from Orange02 cells during growth in batch cultures, 20% v/v sunflower oil, which is a proper solvent

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Table 6. PDR transcription factors regulating expression of genes induced 1.5-fold or more in strain Orange02

	Pdr1	Pdr3	Pdr8	Yrr1	Ym1	PDRE sites	References
YGR035C	*	*	*	*		0	(DeRisi *et al.*, 2000; Le Crom *et al.*, 2002; Devaux *et al.*, 2002; Hikkel *et al.*, 2003; Tuttle *et al.*, 2003; Lucau-Danila *et al.*, 2003; Onda *et al.*, 2004)
URA1				*		0	(Le Crom *et al.*, 2002)
GRE2	*	*				2	(DeRisi *et al.*, 2000; Nawrocki *et al.*, 2001; Hikkel *et al.*, 2003; Tuttle *et al.*, 2003; Onda *et al.*, 2004)
PDR15	*	*	*			2	(Wolfger *et al.*, 1997; DeRisi *et al.*, 2000; Hikkel *et al.*, 2003; Tuttle *et al.*, 2003)
YLR346C	*	*		*		3	(DeRisi *et al.*, 2000; do Valle Matta *et al.*, 2001; Le Crom *et al.*, 2002; Devaux *et al.*, 2002; Hikkel *et al.*, 2003; Tuttle *et al.*, 2003; Lucau-Danila *et al.*, 2003; Onda *et al.*, 2004)
PDR5	*	*	*			6	(DeRisi *et al.*, 2000; Miura *et al.*, 2001; Devaux *et al.*, 2002; Teixeira and Sa-Correia, 2002; Tuttle *et al.*, 2003; Lucau-Danila *et al.*, 2003; Onda *et al.*, 2004; Wehrschutz-Sigl *et al.*, 2004)
ICT1	*	*				1	(DeRisi *et al.*, 2000; Le Crom *et al.*, 2002; Devaux *et al.*, 2002; Hikkel *et al.*, 2003; Tuttle *et al.*, 2003; Lucau-Danila *et al.*, 2003; Onda *et al.*, 2004)
YPL088W	*	*		*		1	(DeRisi *et al.*, 2000; Nawrocki *et al.*, 2001; do Valle Matta *et al.*, 2001; Le Crom *et al.*, 2002; Devaux *et al.*, 2002; Tuttle *et al.*, 2003; Lucau-Danila *et al.*, 2003; Onda *et al.*, 2004)
FLR1		*		*		0	(Broco *et al.*, 1999; Devaux *et al.*, 2002)
PDR10	*	*				2	(Wolfger *et al.*, 1997; Onda *et al.*, 2004)
MIG2	*					1	(Miura *et al.*, 2001)
YKL071W	*	*				0	(Devaux *et al.*, 2002; Tuttle *et al.*, 2003; Onda *et al.*, 2004)
VHR1	*					3	(Lucau-Danila *et al.*, 2003)
TPO1	*	*				1	(DeRisi *et al.*, 2000; do Valle Matta *et al.*, 2001; Tuttle *et al.*, 2003; Onda *et al.*, 2004)
PDR3		*				5	(Devaux *et al.*, 2002)
SNQ2	*	*	*	*	*	3	(DeRisi *et al.*, 2000; Le Crom *et al.*, 2002; Devaux *et al.*, 2002; Hikkel *et al.*, 2003; Tuttle *et al.*, 2003; Lucau-Danila *et al.*, 2003; Onda *et al.*, 2004; Wehrschutz-Sigl *et al.*, 2004)

List of genes induced more than 1.5-fold in strain Orange02 that are known to be regulated by the transcription factors Pdr1, Pdr3, Pdr8, Yrr1 or Yrm1. DNA microarray data of four individual CEN.PK113-7D cultures and three individual Orange02 cultures were analysed. Of 36 genes that were induced 1.5-fold or more, 16 have been reported to be regulated by one or more of the above-mentioned transcription factors as indicated by an asterisk. The references in which these documented regulations are described are indicated. The database YEASTRACT was used in this search (Teixeira *et al.*, 2006).

for β-carotene, was added to the growth medium. Reference strain CEN.PK113-7D and Orange02 were grown for 72 h in batch cultures. After centrifuging the cultures, it was observed that the sunflower oil layer added to strain Orange02 displayed a yellow colour, whereas the colour of the sunflower oil layer added to CEN.PK113-7D remained transparent (Figure 4). The same experiment was also performed using strains that naturally produce carotenoids; a *X. dendrorhous* wild-type strain, which mainly accumulates astaxanthin and a β-carotene-accumulating mutant of *X. dendrorhous* (Girard *et al.*, 1994). Growing these *X. dendrorhous* strains in the presence of sunflower oil did not result in colouring of the oil phase (Figure 4). HPLC studies showed that 0.14 µg/ml β-carotene ($\pm$0.06, $n = 3$) was present in the sunflower oil fraction added to strain Orange02. No other carotenoids were detected in the oil fraction. In the sunflower oil fractions of the reference *S. cerevisiae* strain and the *X. dendrorhous* strains, no carotenoid accumulation was detected. The observation that only β-carotene was measured was surprising, because strain Orange02 also accumulates other carotenoids, especially phytoene, during growth in batch cultures (Table 4). The induced expression of multidrug resistance transporters in strain Orange02, and the presence of β-carotene in the sunflower oil fraction added to the growth medium of strain Orange02, makes it tempting to suggest that multidrug resistance transporters are involved in transport and secretion of β-carotene specifically out of carotenoid-producing *S. cerevisiae* cells.

Deletion of *pdr10* results in decreased growth and carotenoid production levels in carotenoid-producing cells

To determine whether deletion of multidrug resistance transporters identified in the DNA microarray experiments of Orange02 would result in sensitivity towards β-carotene production, an episomal vector containing carotenogenic genes and empty vector as control were transformed into BY4741 strains individually deleted for *pdr5, pdr10, pdr15, snq2, tpo1* or *flr1* and into a BY4741 reference strain. The transformation efficiency for the empty vector YEplac195, 4000 transformants/µg DNA, was similar for all strains. Transformation of the carotenogenic plasmid YEplac195 YB/I/E yielded comparable efficiency (2000 transformants/µg DNA) in the reference and *pdr5, pdr15, snq2, tpo1* or *flr1* deletion strains, but was far lower for the strain deleted for *pdr10* (50 transformants/µg DNA). Furthermore, the reference and *pdr5, pdr15, snq2, tpo1* or *flr1* deletion strains yielded 100%

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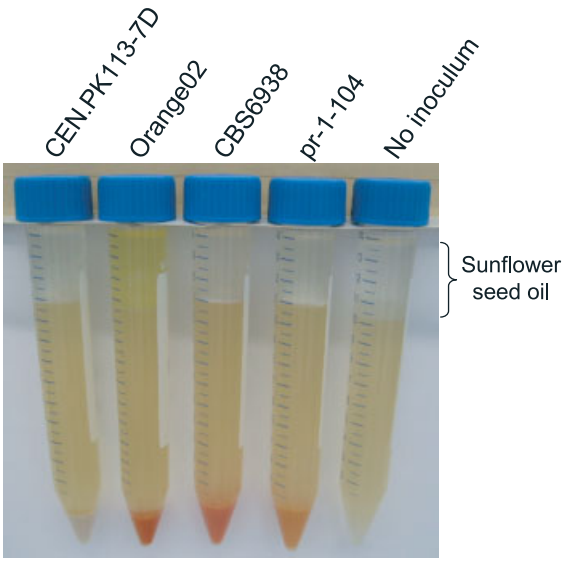

Figure 4. Growth of strain Orange02 in the presence of sunflower oil in batch cultures results in colouring of the oil fraction. *S. cerevisiae* strains CEN.PK113-7D (reference) and Orange02 (YB/I/E), and *X. dendrorhous* strains CBS6938 (reference) and PR-1-104 (β-carotene-accumulating mutant) were grown in batch cultures for 72 h in the presence of 20% v/v sunflower oil. After centrifuging and photographing the cells, HPLC was performed to determine the presence of carotenoids in the sunflower oil added to the cells

orange-coloured transformants upon transformation with YEplac195 YB/E/I, whereas only 25% of the *pdr10* deletion transformants had an orange colour. These results were obtained from three independent transformations, showing that the effect of decreased transformation of the strain deleted for pdr10 with carotenogenic genes is reproducible. To determine whether growth rates and carotenoid production levels were affected, coloured transformants of the reference strain and cells deleted for *pdr5* or *pdr10* were grown in liquid medium. A control experiment with strains carrying YEplac195 empty vector control was included. Introduction of carotenoid production into the reference and *pdr5* deletion strain yielded strains with similar growth properties (Table 7). Introduction of carotenoid production into the strain deleted for *pdr10* decreased the growth rate by 25% compared to the reference and *pdr5* deletion strains. This was accompanied by a 60% decrease of total carotenoid levels in a *pdr10* deletion mutant when compared to the reference strain, whereas carotenoid accumulation was unaffected in the strain deleted for *pdr5* (Table 7). This severe drop in carotenoid synthesis might reflect a loss of part of the episomal expression cassette, which would correlate with the loss of coloration of three-quarters of the Δ*pdr10* YEplac195 YB/I/E transformants. These results clearly demonstrate the correlation between the absence of Pdr10 and the increased sensitivity to carotenoid synthesis in *S. cerevisiae*.

Discussion

This work describes a systematic investigation of the effects of heterologous metabolite production, in this case carotenoids, on the physiology and transcriptional response of *Saccharomyces cerevisiae*. For this purpose, two complementary techniques were combined: chemostat cultivation and genome-wide transcriptome analysis using DNA microarrays. Two different strains producing low (Orange01) and high (Orange02) amounts of carotenoids were grown and analysed. With the exception of oscillatory behaviour in strain Orange02, which was prevented by the addition of a low amount of ethanol to the culture medium, the recorded parameters were consistent, which suggested that carotenoid production did not affect yeast physiology to a large extent. However, it was noticed that the amount and distribution of

Table 7. Maximum specific growth rates (μ_{max}, h^{-1}) and intracellular accumulation of total carotenoids of BY4741, BY4741Δ*pdr5* and BY4741Δ*pdr10* transformed with YEplac195 empty vector or YEPplac195 YB/I/E

	Growth rates (μ_{max}, h^{-1})			Total carotenoid production (μg/g dw)	
Strain	**+ Empty vector**	**+ YB/I/E**	$\mu_{YB/I/E}/\mu_{empty\ vector}$	**+ Empty vector**	**+ YB/I/E**
BY4741	0.35 ± 0.01	0.31 ± 0.01	0.88	0	612 ± 51
Δpdr5	0.36 ± 0.02	0.34 ± 0.01	0.93	0	592 ± 39
Δpdr10	0.35 ± 0.01	0.23 ± 0.02	0.67	0	247 ± 52

Cells were inoculated at similar optical densities and grown on synthetic glucose medium in batch cultures in triplicate.

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carotenoid intermediates could be influenced by the culture conditions. The Orange01 strain showed a higher β-carotene accumulation in chemostat culture than in batch culture. An obvious difference between these two cultivation modes is the type of metabolism employed. In chemostat culture yeast metabolism is exclusively respiratory, while in batch culture yeast metabolism is respiro-fermentative. Unfortunately, this explanation does not stand for Orange02, which shows an opposite behaviour, exhibiting a 3.5-fold reduction in β-carotene accumulation in chemostat compared to batch cultivation. Nonetheless the main difference between the two strains remained the large accumulation of phytoene and, to a lower extent, lycopene in the Orange02 strain. The high concentrations of these compounds may also influence the accumulation of the other intermediates. Furthermore, the difference in the transcriptional response between the two strains may originate from the difference of carotenoid intermediate profiles.

Genome-wide transcriptional analysis of strain Orange02 resulted in the identification of a specific stress response, comprising a set of genes known to be involved in pleiotropic drug resistance (PDR). This observed response suggests that carotenoid production above a certain concentration is toxic for the yeast cell. The induction of multidrug resistance transporters, which are known to be involved in the secretion of hundreds of structurally and functionally unrelated compounds in carotenoid-producing yeast strains, suggests that the transporters are involved in secretion of the produced carotenoids. Furthermore, the observed repression of *PAU* genes, which encode proteins localized in the cell wall, suggests that the cell wall structure is changed in response to carotenoid production. This response might occur to facilitate secretion of carotenoids. To date, only one report has described the secretion of carotenoids from a carotenoid-producing organism (Hirschberg and Harker, 1999). As determined by electron microscopy, the Gram-negative coccus *Paracoccus marcusii* produces lipophilic carotenoid-containing vesicles intracellularly. These vesicles move across the plasma membrane, cover the surfaces of cells and form a separate entity that is subsequently released into the growth medium (Hirschberg and Harker, 1999). Direct secretion of carotenoid-containing vesicles was not observed in the carotenoid-producing *S. cerevisiae* strains, as the growth medium did not change colour. Only after the addition of sunflower oil, which is a hydrophobic solvent in which β-carotene dissolves easily, was accumulation of β-carotene in liquid phase measured. This accumulation might be the result of active or passive secretion of β-carotene out of the recombinant cells, mediated by the drug resistance transporters identified in the microarray experiments.

Possibly high production levels of carotenoids cause membrane stress (Gruszecki and Strzalka, 2005). Previous experiments have shown that ABC transporters are able to translocate membrane phospholipids and sterols and function in controlling or modulating membrane lipid homeostasis, regulation of membrane permeability and phospholipid bilayer distribution (Wilcox *et al*., 2002; Li and Prinz, 2004). In *Saccharomyces cerevisiae* cells producing high levels of carotenoids, the membrane buffering capacity to accumulate the produced carotenoids may not be sufficient. A perturbation of the membrane structure by accumulation of carotenoids might cause membrane stress and induce the expression of PDR transporters. Indeed, a recent study has shown that compounds with potential membrane-damaging or perturbing effects might function as an activating signal for Pdr1 and Pdr3, which suggests a role for their target genes, like the PDR transporters identified in our study, in membrane lipid organization or remodelling (Schuller *et al*., 2007). The authors suggest that certain yeast ABC transporters, such as Pdr5, Pdr10 and Pdr15, may guard and maintain membrane bilayer function by binding and removing unwanted or toxic lipids (Schuller *et al*., 2007). Our results suggest that a cellular response to relieving membrane stress caused by accumulation of carotenoids could be mediated by the multidrug resistance transporters encoded by *PDR5, PDR10, PDR15, SNQ2* and the major facilitator transporters encoded by *TPO1* and *FLR1*. These transporters might bind, remove and secrete carotenoids from membranes, consistent with the suggested role for multidrug resistance transporters in membrane lipid organization or remodelling (Schuller *et al*., 2007). As shown by our results and in other studies, Pdr10 could play a more crucial role (Rockwell *et al*., 2009). Of course, other, yet-unknown mechanisms besides membrane stress are conceivable.

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The observed pleiotropic drug resistance response might be a general feature in the production of heterologous compounds derived from the ergosterol biosynthetic pathway, such as taxol, long-chain polyunsaturated fatty acids or flavonoids (Chemler *et al.*, 2006). Interestingly, Ro *et al.* (2008) found similar effects studying the synthesis of artemisinic acid in *S. cerevisiae*. Production of this isoprenoid led to a larger number of genes being up- and downregulated genes compared to this study, but shared some of the pleiotropic drug resistance genes that were also found in our study. Therefore, these genes may be attractive targets for biotechnological production of isoprenoids in *S. cerevisiae*.

Our results also show that the addition of a suitable solvent to *S. cerevisiae* cells producing isoprenoid compounds might result in secretion and accumulation of the product in the added solvent. Overall production levels might be enhanced when potentially toxic metabolites are secreted out of the cells. Secretion of isoprenoids could offer a great advantage towards future biotechnological production in *Saccharomyces cerevisiae* and in other organisms.

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