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Carotenoid accumulation in bacteria with enhanced supply of isoprenoid precursors by upregulation of exogenous or endogenous pathways

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ABSTRACT

Carotenoids are isoprenoid pigments of industrial and nutritional interest. Although they are produced in non-carotenogenic *Escherichia coli* engineered with the appropriate biosynthetic genes, only a limited pool of their metabolic precursors is available in these bacteria. We have compared the production of carotenoids (lycopene) in strains in which the supply of precursors was enhanced either by upregulating the endogenous pathway via overexpression of deoxyxylulose 5-phosphate synthase (DXS) or by incorporating an exogenous MVA+ operon. In strains expressing DXS under the control of a leaky IPTG-inducible promoter, lycopene accumulation was increased up to 8-fold in the absence of inducer. Addition of IPTG, however, negatively affected lycopene production. Although induction of too high levels of the MVA+ operon enzymes also appeared to cause interference with cell metabolism, supplementation with mevalonate (to be metabolized into carotenoid precursors) resulted in a 10-fold increase in lycopene levels in cells with a near wild-type background. An additional 2-fold increase (up to 228 mg/l) was obtained using an engineered BL21 strain. These results confirm that the MVA+ pathway is most convenient to upregulate the production of carotenoids (lycopene) production in *E. coli* but that factors other than precursor supply should be considered for high pigment accumulation levels.

1. Introduction

Carotenoids are an abundant group of isoprenoid pigments synthesized by all photosynthetic organisms and some non-photosynthetic bacteria and fungi. Animals do not synthesize carotenoids de novo but take them from their diet as an essential source of vitamin A and retinoids. Carotenoids such as lycopene also act as natural antioxidants that help to prevent some types of human cancer and degenerative diseases (Fraser and Bramley, 2004). Besides their activity as pro-vitamin A and their potential as nutraceuticals, the colorant and aromatic properties of some carotenoids and derived compounds have an important industrial value (Bartley and Scolnik, 1995; Hirschberg, 2001; Sandmann, 2001; Fraser and Bramley, 2004). Most commercial carotenoids are obtained by chemical synthesis, but the growing demand of natural additives has spurred several metabolic engineering approaches to increase carotenoid production in plants and microorganisms (Lee and Schmidt-Dannert, 2002; Fraser and Bramley, 2004; Botella-Pavía and Rodríguez-Concepción, 2006). Carotenoids have been successfully produced even in non-carotenogenic bacteria such as *Escherichia coli* after transformation with appropriate genes from carotenogenic organisms (Fig. 1). However, important problems remain to be solved to efficiently overproduce carotenoids in non-carotenogenic hosts. One of such problems is the supply of their metabolic precursors.

Structurally carotenoids are tetraterpenes derived from the 5-carbon units isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP), the universal precursors of all isoprenoid compounds (Cunningham and Gantt, 1998). IPP and DMAPP can be interconverted by the enzyme IPP/DMAPP isomerase (IDI). The addition of three molecules of IPP to one DMAPP unit catalyzed by the enzyme geranylgeranyl diphosphate (GGPP) synthase (GDS) yields GGPP, the immediate precursor for carotenoids (Fig. 1). There are two pathways for the synthesis of these prenyl diphosphates: the mevalonic acid (MVA) pathway and the methylerythritol 4-phosphate (MEP) pathway (Lichtenthaler, 1999; Rodríguez-Concepción and Boronat, 2002; Eisenreich et al., 2004). Archaebacteria, fungi and animals synthesize their isoprenoids exclusively through the operation of the MVA

Abbreviations: DXP, 1-deoxy-D-xylulose 5-phosphate; DXS, DXP synthase; MEP, 2-C-methyl-D-erythritol 4-phosphate; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; GGPP, geranylgeranyl diphosphate; MVA, mevalonic acid.

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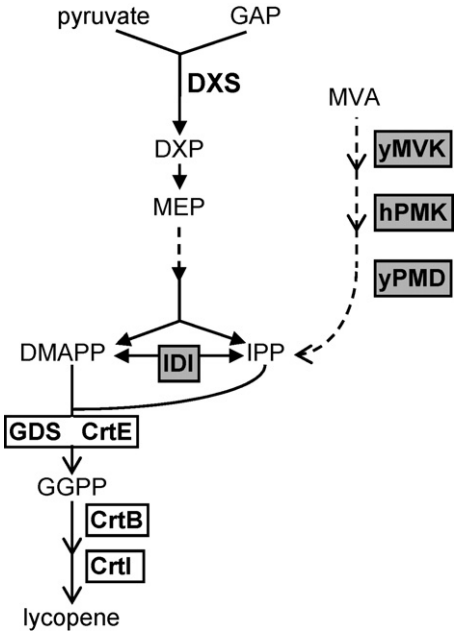

Fig. 1. Pathways involved in lycopene biosynthesis in engineered *E. coli* cells. GAP, D-glyceraldehyde 3-phosphate; DXP, 1-deoxy-D-xylulose 5-phosphate; MEP, 2-*C*-methyl-D-erythritol 4-phosphate; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; GGPP, geranylgeranyl diphosphate; MVA, mevalonic acid; DXS, DXP synthase. The enzymes encoded by the MVA+ operon are boxed in gray: yMVK, yeast mevalonate kinase; hPMK, human 5-phosphomevalonate kinase; yPMD, yeast 5-diphosphomevalonate decarboxylase; IDI, *E. coli* IPP/DMAPP isomerase. The enzymes encoded by the pLYC plasmid are boxed in white: CrtE, GGPP synthase (GDS); CrtB, phytoene synthase; CrtI, phytoene desaturase.

pathway, whereas the MEP pathway is the only one present in most eubacteria, including *E. coli* (revised in Lichtenthaler, 1999; Rodríguez-Concepción and Boronat, 2002).

The first reaction of the MEP pathway (Fig. 1) is the production of deoxyxylulose 5-phosphate (DXP) from pyruvate and glyceraldehyde 3-phosphate, catalyzed by DXP synthase (DXS). Many studies have reported a positive correlation between DXS levels and carotenoid production in bacteria (Albrecht et al., 1999; Harker and Bramley, 1999; Cunningham et al., 2000; Jones et al., 2000; Matthews and Wurtzel, 2000; Kim and Keasling, 2001; Kang et al., 2005; Yuan et al., 2006) and plants (Estévez et al., 2001; Botella-Pavía et al., 2004; Enfissi et al., 2005; Carretero-Paulet et al., 2006). Furthermore, upregulation of DXS levels also results in increased accumulation of other MEP-derived isoprenoids such as monoterpetenes, diterpenes, sesquiterpenes, and the prenyl tail of ubiquinone in bacteria (Harker and Bramley, 1999; Martin et al., 2003; Reiling et al., 2004; Kim et al., 2006) and chlorophylls, tocopherols, taxadiene, cytokinins, gibberellins, and essential oils in plant cells (Estévez et al., 2001; Botella-Pavía et al., 2004; Carretero-Paulet et al., 2006; Morris et al., 2006; Muñoz-Bertomeu et al., 2006), confirming that the effect of DXS overexpression on carotenoid levels is due to an enhanced production of their metabolic precursors. Although increasing the levels of other individual MEP pathway enzymes has sometimes been reported to result in enhanced carotenoid production in *E. coli* cells, the increase was always lower than that achieved by DXS overexpression (Albrecht et al., 1999; Cunningham et al., 2000; Miller et al., 2000; Kim and Keasling, 2001; Yuan et al., 2006). DXS therefore appears to be the most appropriate enzyme to overexpress in order to increase the production of IPP and DMAPP by the endogenous MEP pathway in *E. coli*. Activities downstream the MEP pathway such as IDI and GDS (Fig. 1) have also been shown to be limiting for carotenoid synthesis in engineered *E. coli* cells (Kajiwara et al., 1997; Albrecht et al., 1999; Wang et al., 1999; Cunningham et al., 2000).

Disruption of the gene encoding DXS or any other MEP pathway enzyme is lethal in *E. coli* cells but it can be rescued by engineering a synthetic operon harboring heterologous MVA pathway genes required to transform exogenously supplied MVA into IPP (Campos et al., 2001) (Fig. 1). The endogenous IDI activity of *E. coli* can produce DMAPP from MVA-derived IPP. Nevertheless, the introduction of the bacterial *idi* gene into the synthetic operon to create the MVA+ operon resulted in improved growth, likely because the production of high IPP levels is toxic for bacterial cells (Campos et al., 2001; Martin et al., 2003). The MVA+ operon, under the transcriptional control of the arabinose-induced P_{BAD} promoter, is amenable to a high degree of control (Campos et al., 2001). A similar operon was shown to produce very high levels of IPP and DMAPP molecules from the MVA added to the growth medium and to outperform DXS overexpression for improving amorphadiene production in bacteria (Martin et al., 2003). It is not known, however, to what extent the accumulation of carotenoids in *E. coli* cells is limited by the supply of isoprenoid precursors. Here we have standardized the experimental conditions with *E. coli* cells harboring the MVA+ operon and a plasmid to synthesize lycopene from IPP and DMAPP to compare the accumulation of lycopene after upregulating the supply of precursors via the endogenous MEP pathway or the synthetic MVA+ operon.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Plasmids and strains used in this study are included in Table 1. The MVA+ synthetic operon from strain EcAB4-1 (Sauret-Güeto et al., 2006) was incorporated into the genome of the BL21 (DE3) strain by phage P1 transduction to construct strain EcAM5-1. Competent cells were transformed with plasmid pACCRT-EIB (here designated pLYC) harboring the *Erwinia uredovora* (currently named *Pantoea ananatis*) *crtE*, *crtB* and *crtI* genes for lycopene synthesis (Cunningham et al., 1993) and, when indicated, plasmid pTAC-ORF2 (here designated pTAC-DXS) containing the *E.*

Table 1
Plasmids and strains used in this study

Name	Description	Antibiotic marker	Reference
Plasmids			
pLYC	pACCRT-EIB, with *crtE*, *crtB* and *crtI* genes	Chloramphenicol	Cunningham et al. (1993)
pTAC-DXS	pTAC-ORF2, with a IPTG-inducible *dxs* gene	Ampicillin	Lois et al. (1998)
Strains			
K12 MG1655	F-lambda-*ilvG-rfb*-50 *rph*-1	None	Blattner et al. (1997)
EcAB4-1	K12 MG1655 with an arabinose-inducible MVA+ operon	Kanamycin	Sauret-Güeto et al. (2006)
BL21(DE3)	B F-*dcm ompT hsdS*{rB-mB-) *gal* λ(DE3)	None	Stratagene
EcAM5-1	BL21(DE3) with an arabinose-inducible MVA+ operon	Kanamycin	This work

coli dxs gene under the control of an IPTG-inducible promoter (Lois et al., 1998). All strains were grown at 37 °C in Luria broth (LB) medium supplemented with antibiotics to final concentrations of 25 μg/ml kanamycin (Km, to select for the MVA+ operon), 17 μg/ml chloramphenicol (Cm, to select for the pLYC plasmid) or/and 100 μg/ml ampicillin (Ap, to select for the pTAC-DXS plasmid). When indicated, the growth medium was supplemented with arabinose and MVA prepared as described (Campos et al., 2001). Bacterial growth in liquid media was monitored by measuring optical density at 600 nm (OD_{600}). When required, the culture was diluted to enable photometric measurement in the linear range (OD_{600} = 0.1–0.5).

2.2. Quantification of lycopene production

Fresh LB medium containing the appropriate supplements was inoculated with 0.01 volumes of overnight cultures of several independent colonies from each *E. coli* strain and grown at 37 °C. After quantifying cell growth (OD_{600}), a 1.3 ml aliquot of the culture was centrifuged at 13,000 × *g* for 3 min and the cell pellet was resuspended in 700 μl of acetone. The samples were incubated at 55 °C for 15 min in the dark and then centrifuged at 13,000 × *g* for 10 min to recover the supernatant with the pigment, which was placed in a clean tube. At least two samples were taken from each culture for lycopene extraction. When necessary, up to three extractions in acetone were performed to remove all color from the cell pellet of a single sample. In this case, the supernatants of the different extractions were pooled. Lycopene was quantified as described (Harker and Bramley, 1999) by measuring absorbance at 472 nm of diluted supernatants in acetone and comparing with a standard curve made with known concentrations of a lycopene standard (Sigma).

3. Results and discussion

3.1. Induction of too high DXS levels negatively impacts lycopene accumulation in E. coli

The most direct strategy to increase the supply of precursors for carotenoid production in *E. coli* is engineering the endogenous MEP pathway. Although increased levels of several MEP pathway enzymes has been reported to result in enhanced carotenoid production in *E. coli* cells, the increase was always highest with DXS (Albrecht et al., 1999; Cunningham et al., 2000; Miller et al., 2000; Kim and Keasling, 2001; Yuan et al., 2006), supporting that DXS catalyzes the main flux-controlling step of the MEP pathway. We therefore selected DXS as the most appropriate enzyme to overexpress in order to efficiently upregulate the endogenous MEP pathway for the production of IPP and DMAPP in *E. coli*.

E. coli K12 MG1655 cells with a genomic copy of the MVA+ operon (strain EcAB4-1) (Sauret-Güeto et al., 2006) were transformed with the pLYC plasmid harboring the *Erwinia uredovora* genes required to synthesize lycopene from IPP and DMAPP (Table 1 and Fig. 1). Successful transformants were identified based on their double antibiotic resistance and the red color imparted by lycopene production. The presence of the *E. coli idi* gene in the MVA+ operon (Campos et al., 2001) and the *E. uredovora crtE* gene in the pLYC plasmid (Cunningham et al., 1993) ensured that carotenoid production was not limited by the endogenous IDI or GDS activities in EcAB4-1 pLYC cells. Competent EcAB4-1 pLYC cells were then transformed with the pTAC-DXS plasmid (Table 1), in which the expression of the *E. coli dxs* gene is under the control of an IPTG-inducible promoter (Lois et al., 1998), or an empty pTAC vector. Several overnight cultures of pigmented colonies of each strain in media without IPTG were used to inoculate fresh liquid media and quantify the production of lycopene in the presence or absence of IPTG.

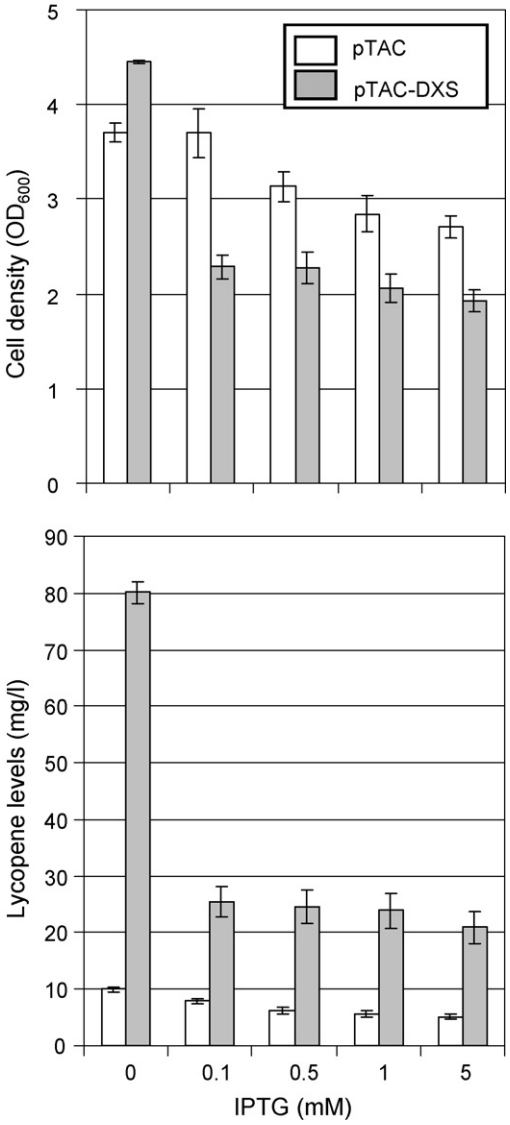

Fig. 2. Effect of DXS overexpression on cell growth and lycopene levels in EcAB4-1 pLYC cells. Liquid media (10 ml) supplemented with the indicated concentrations of IPTG was inoculated with 0.1 ml of overnight cultures of EcAB4-1 pLYC cells harboring the pTAC-DXS plasmid (gray columns) or an empty pTAC vector (white columns) and grown overnight at 37 °C. Cell growth was determined by optical density at 600 nm (OD_{600}) and lycopene content was calculated by measuring the absorbance at 472 nm of acetone extracts of the cells and comparison with a standard curve. Mean values and standard errors corresponding to four independent experiments with several replicas each are represented.

In the absence of IPTG, overnight cultures of EcAB4-1 pLYC pTAC-DXS cells accumulated 8-fold higher lycopene levels compared to control cells harboring the pTAC plasmid (Fig. 2). Upregulation of DXS levels in *E. coli* cells using different expression vectors have been reported to result in typically 2–5-fold increases in lycopene accumulation (Harker and Bramley, 1999; Cunningham et al., 2000; Jones et al., 2000; Kim and Keasling, 2001; Reiling et al., 2004; Kang et al., 2005). The use of the pTAC-DXS construct, however, was shown to lead to 7-fold higher lycopene levels in *E. coli* colonies grown in the absence of IPTG (Matthews and Wurtzel, 2000), an increase similar to that reported here in liquid cultures (Fig. 2). The pTAC vectors lack tight control of the basal expression level from the strong P_{tac} promoter, leading to DXS expression in the absence of induction by IPTG. Addition of IPTG to the growth media, however, resulted in much lower increases in lycopene

accumulation (Fig. 2). Low concentrations of the inducer (0.1 mM) had no effect on the growth of control EcAB4-1 pLYC pTAC cells but it dramatically reduced growth of EcAB4-1 pLYC pTAC-DXS cells to 50% compared to that in the absence of IPTG (Fig. 2). Addition of higher concentrations of IPTG progressively decreased growth of both strains (Fig. 2). The strong negative effect of IPTG on cell growth and lycopene production in *E. coli* cells expressing DXS under a strong inducible promoter has been repeatedly observed by other authors using different strains and constructs (Matthews and Wurtzel, 2000; Jones et al., 2000; Kim and Keasling, 2001). It has been suggested that production of excess DXS levels might divert a substantial amount of its substrates pyruvate and glyceraldehyde 3-phosphate to the MEP pathway and prevent their use in other central metabolic pathways. This, together with the energetic requirements associated to the production of high protein levels, might compromise the energetic and metabolic resources of the cell. Additionally, the production of a single molecule of lycopene requires 8 molecules of pyruvate, 8 glyceraldehyde 3-phosphate, 8 CTP, 8 ATP, and 16 NADPH (Alper et al., 2005) and the development of storage structures for such a lypophylic compound is also expensive in energetic terms. It is therefore expected that the metabolic burden likely caused by DXS overexpression has a negative impact on the production and accumulation of lycopene. Together, our data show that high increases in lycopene production can be achieved in EcAB4-1 pLYC pTAC-DXS cells, which accumulated up to 80 mg of lycopene per liter of culture. However, manipulation of the endogenous MEP pathway in *E. coli* by altering DXS levels may have effects on cell metabolism that are difficult to control, resulting in suboptimal production of lycopene.

3.2. An exogenous supply of isoprenoid precursors dramatically increases lycopene accumulation

To better determine the influence of highly increased IPP and DMAPP levels on the production of lycopene in bacteria, we took advantage of the MVA+ operon. The transformation of exogenously supplied MVA into IPP and DMAPP by the MVA+ operon enzymes was expected to have little or no interference with the endogenous cell metabolism in EcAB4-1 pLYC cells, as bacteria do not produce MVA or have the enzymes to metabolize it into isoprenoid precursors. Because the MVA+ operon is under the control of the arabinose-induced P_{BAD} promoter (Campos et al., 2001), we first tested the influence of the concentration of arabinose in the accumulation of lycopene in overnight EcAB4-1 pLYC cultures. As expected, cells grown in media supplemented with 1 mM MVA and different concentrations of arabinose (from 0.1 to 1%) accumulated more lycopene than cells grown without the inductor (Fig. 3). However, lycopene accumulation was highest at the lowest concentration of arabinose tested (0.1%), reaching levels that were ca. 4-fold higher than those measured in the absence of inducer. No effect on cell growth was observed at this concentration of the inductor. Although increasing concentrations of arabinose did have an inhibitory effect on cell growth, the decrease in cell growth was not as dramatic as that observed for lycopene accumulation (Fig. 3). Both total and normalized (in a per cell basis) lycopene levels decreased in an arabinose concentration-dependent manner. It is therefore possible that inducing the production of high levels of MVA+ operon enzymes could actually interfere with cell metabolism. Alternatively, the inhibitory effect on growth and lycopene accumulation observed in EcAB4-1 pLYC cultures grown in the presence of high arabinose concentrations might be derived from the production of excess IPP and DMAPP that cannot be efficiently channeled to the production of lycopene and become toxic for the cell (Campos et al., 2001; Martin et al., 2003).

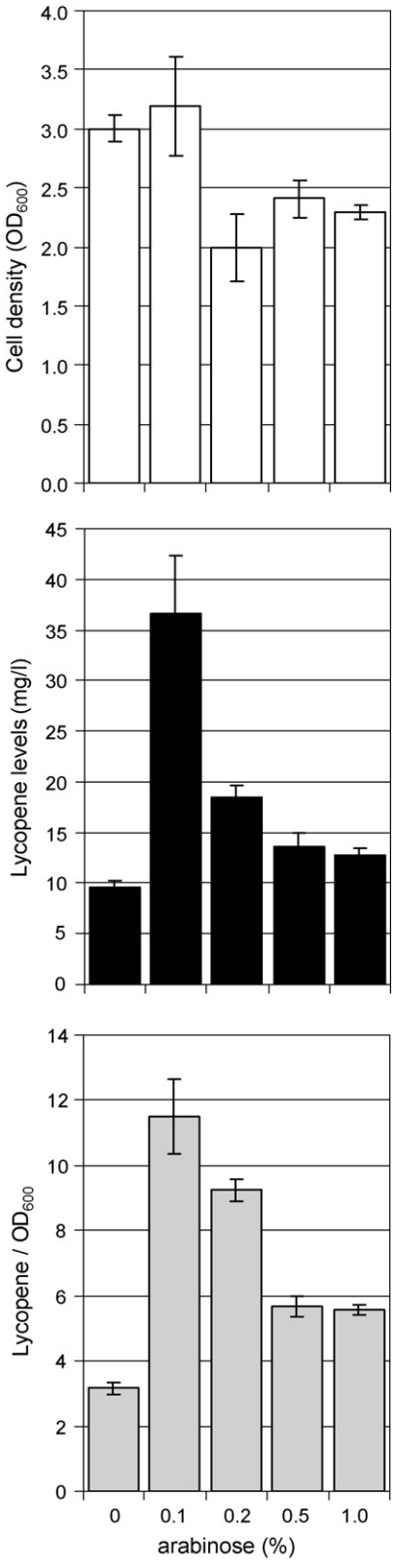

Fig. 3. Influence of arabinose concentration on cell growth and lycopene accumulation in EcAB4-1 pLYC cultures. Liquid media (10 ml) containing 1 mM MVA and different concentrations of arabinose (from 0 to 1%) were inoculated with 0.1 ml of overnight cultures of EcAB4-1 pLYC cells in media lacking arabinose and MVA and grown overnight at 37 °C. Cell growth (white columns) and lycopene content (black columns) were measured as described in Fig. 2. Lycopene levels normalized to cell density are also shown (gray columns). Mean values and standard errors from two replicates of two independent cultures are shown.

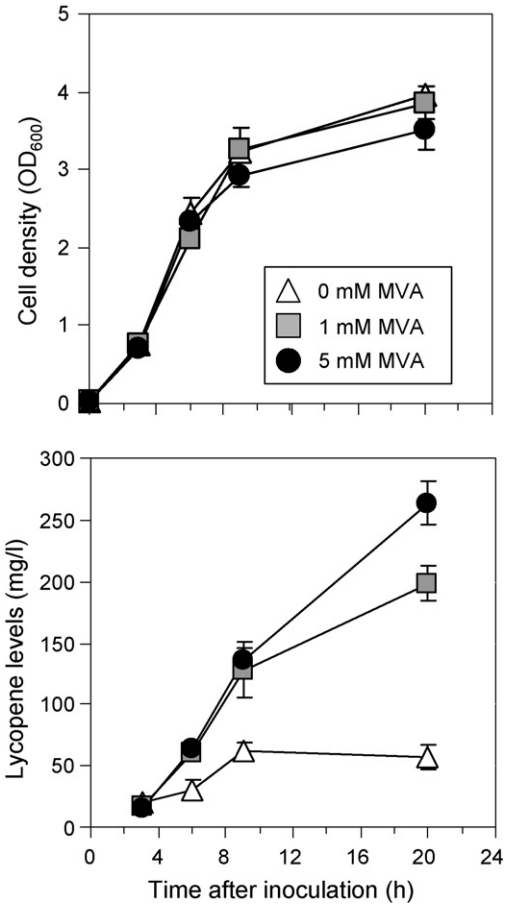

Fig. 4. Time course of cell growth and lycopene accumulation in EcAB4-1 pLYC cultures. Liquid media (100 ml) containing 0.1% arabinose and either supplemented or not with MVA were inoculated with 1 ml of overnight cultures of EcAB4-1 pLYC cells in media lacking arabinose and MVA and grown at 37 °C. At the indicated times, 1 ml samples were collected and used to measure cell growth and lycopene content as described in Fig. 2. Mean values and standard errors from two replicates of two independent cultures are shown.

To distinguish between these two possibilities, we grew EcAB4-1 pLYC cells in media supplemented with 0.1% arabinose and two concentrations of MVA (1 and 5 mM) and monitored the time-course of growth and lycopene accumulation. As shown in Fig. 4, lycopene accumulation in EcAB4-1 pLYC cultures grown in liquid medium supplemented with 0.1% arabinose but no MVA reached a maximum level at the end of the exponential growth phase. When 1 mM MVA was included in the medium, cells accumulated significantly higher lycopene levels during their exponential growth and they continued synthesizing this carotenoid when reaching the stationary phase. No effect was observed in cell growth (Fig. 4), consistent with our previous results (Fig. 3). When the concentration of MVA added to the growth medium was increased to 5 mM, a slight inhibition of growth was observed when cells were reaching the stationary stage (Fig. 4). Lycopene accumulation during the stages of active cell growth was similar in media supplemented with 1 mM or 5 mM MVA. In overnight (20 h) cultures, however, lycopene levels in cultures supplemented with 5 mM MVA were significantly higher than those measured in cultures supplemented with 1 mM MVA (Fig. 4). It is therefore possible that the decrease of cell growth observed in overnight cultures might be caused by a deleterious effect of increased accumulation of hydrophobic lycopene molecules within bacterial cells. Together, the fact that lycopene accumulation was negatively correlated with arabinose concentration in the medium (Fig. 3) but positively correlated with MVA concentration (Fig. 4) supports that excess levels of the enzymes encoded by the MVA+ operon cause interference with cell metabolism and eventually result in a lower production of lycopene. Our results also support that an enhanced production of MVA-derived IPP and DMAPP only marginally affects growth of EcAB4-1 pLYC cells, possibly because a large proportion of these precursors is efficiently channeled to the biosynthesis of lycopene.

We next determined the maximum level of lycopene that could be produced in overnight (20 h) cultures of EcAB4-1 pLYC cells with an excess supply of MVA. As shown in Fig. 5, some inhibition of

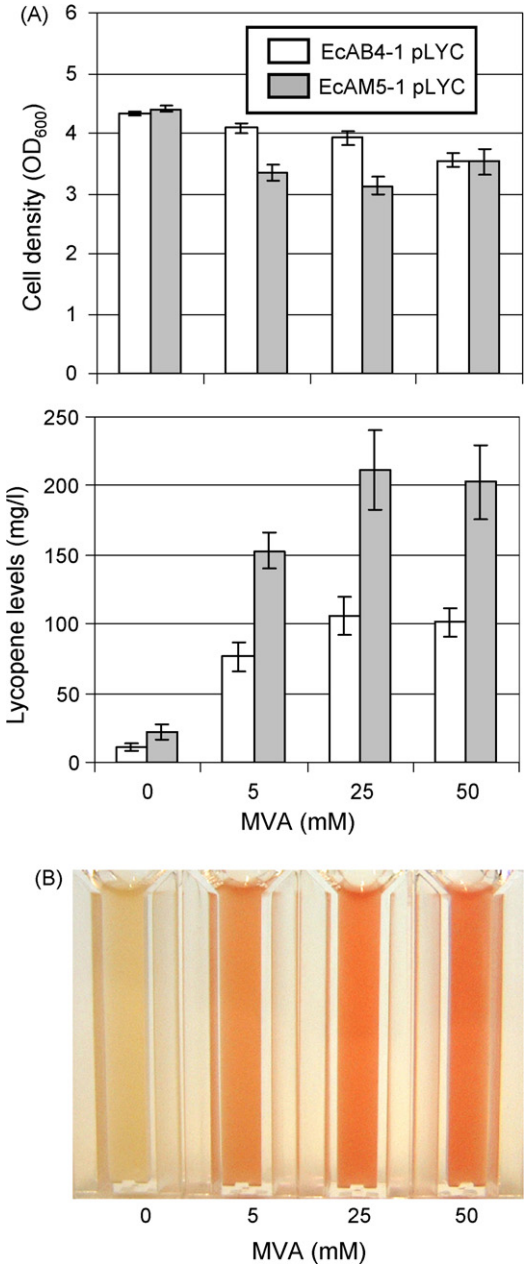

Fig. 5. Cell growth and lycopene levels in MVA-supplemented cultures. Liquid media (10 ml) containing 0.1% arabinose and the indicated concentrations of MVA were inoculated with 0.1 ml of overnight cultures of EcAB4-1 pLYC (white columns) or EcAM5-1 pLYC (gray columns) strains in media lacking arabinose and MVA and grown overnight at 37 °C. Cell growth and lycopene levels (A) were measured as described in Fig. 2. Mean values and standard errors correspond to two replicates of at least four independent cultures. Aliquots of EcAM5-1 pLYC cultures grown in the presence of the indicated concentrations of MVA are also shown (B).

cell growth was observed as the amount of MVA added to the growth medium increased. By contrast, the production of lycopene in overnight EcAB4-1 pLYC cultures was highly increased with MVA concentration up to 25 mM, reaching levels that were 10-fold higher than those measured in the absence of MVA (Fig. 5). No significant increases were observed when MVA concentration was doubled to 50 mM, suggesting that factors other than precursor supply might be limiting lycopene production under these conditions. Since only a fraction of the supplied MVA appears to be eventually metabolized into lycopene (Yoon et al., 2006), improved production of lycopene by increasing addition of MVA to the growth medium might be at least in part due to a facilitated import into *E. coli* cells (which do not have a transport system for MVA) by simple diffusion.

3.3. Further increases in lycopene accumulation can be achieved by optimizing factors other than precursor supply

Because the genetic background can substantially influence lycopene accumulation in *E. coli* cells (Wurtzel et al., 1997; Kim and Keasling, 2001; Vadali et al., 2005; Yuan et al., 2006), we investigated the production of lycopene in a different *E. coli* strain. When the MVA+ operon was transduced from the K12 MG1655 wild-type background of the EcAB4-1 strain to BL21 (DE3) cells to create strain EcAM5-1 (Table 1), a significant increase in lycopene levels (ca. 2-fold) was observed in EcAM5-1 pLYC cells compared to EcAB4-1 pLYC cells at all the concentrations of MVA tested (Fig. 5). Similarly to that shown for EcAB4-1 pLYC, however, lycopene levels in EcAM5-1 pLYC cells reached a maximum at 25 mM MVA (Fig. 5). These results confirm that the enzymes expressed in *E. coli* cells harboring the pLYC plasmid can channel an enhanced supply of metabolic precursors (IPP and DMAPP) to the production of lycopene until other factors become limiting for its accumulation.

A question arising from our results is why carotenoid accumulation in the BL21 background of the EcAM5-1 strain was improved relative to the near wild-type background of EcAB4-1 cells. No obvious correlation has been found between genetic markers and carotenoid accumulation (Wurtzel et al., 1997). However, because the K12 MG1655 strain has been maintained with minimal genetic modifications, whereas BL21 is the most widely used host background for overexpression of foreign recombinant proteins and is deficient in both Lon and OmpT proteases, it is likely that the mutations in EcAM5-1 cells result in a more appropriate expression of the heterologous genes introduced in the MVA+ operon and the pLYC plasmid or/and an increased stability of the encoded proteins compared to EcAB4-1. Alternatively, EcAM5-1 cells might be able to more efficiently meet the metabolic (energy) and physical (storage) requirements associated with an upregulated production of lycopene. Attempts to control and optimize the production of the enzymes required to synthesize lycopene from its prenyl diphosphate precursors have shown that low levels of such enzymes are enough for efficient lycopene synthesis in rich media, whereas too high levels can actually be deleterious for cell growth and lycopene accumulation (Ruther et al., 1997; Yoon et al., 2006; Yoon et al., 2007). Much less is known about how carotenoid storage can be improved in bacteria.

Our results confirm that engineering an exogenous pathway for enhanced IPP and DMAPP biosynthesis results in higher carotenoid (lycopene) production and less interference with the metabolism of *E. coli* cells when compared to upregulating the levels of enzymes (DXS) of the endogenous MEP pathway. Under our growth conditions, EcAM5-1 pLYC cells accumulated up to 228 mg of lycopene per liter of overnight culture, an impressive 20-fold increase compared to the levels measured in EcAB4-1 pLYC cells grown in the absence of MVA (Fig. 5). Further optimization of growth conditions, vectors, and host strains should result in even higher production of lycopene. In any case, the increase in lycopene accumulation observed in EcAB4-1 pLYC and EcAM5-1 pLYC cells grown in the presence of MVA is substantially higher than that reported for *E. coli* DH5α cells harboring a plasmid-bound IPTG-inducible MVA+ operon from *Streptococcus pneumoniae* (Yoon et al., 2006, 2007). The use of a "complete" MVA operon with all the *Streptomyces* genes required to convert acetyl coenzyme-A (produced by the bacteria) into IPP and DMAPP (Takagi et al., 2000) has been found to only result in increased lycopene production in *E. coli* strains in which the acetate pathway is inactivated (Vadali et al., 2005). However, further work is required to establish whether a complete MVA pathway constructed with genes from other species and with a fine control of its expression might allow to significantly increase the production of lycopene in rich media with no added MVA without compromising the metabolic balance of *E. coli* cells.

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