Functional involvement of a deoxy-D-xylulose 5-phosphate reductoisomerase gene harboring locus of *Synechococcus leopoliensis* in isoprenoid biosynthesis

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Abstract The present work aimed to proof the functionality of the non-mevalonate pathway in cyanobacteria. It was intended to isolate the 1-deoxy-D-xylulose 5-phosphate (DXP) reductoisomerase gene (*dxr*), as this gene encodes the enzyme which catalyzes a pathway-specific, indicative step of this pathway. For this purpose, a segment of *dxr* was amplified from *Synechococcus leopoliensis* SAUG 1402-1 DNA via PCR using oligonucleotides for conserved regions. Subsequent hybridization screening of a genomic cosmid library of *S. leopoliensis* with the PCR segment led to the identification of a 26.5 kbp locus on which a *dxr* homologous gene and two adjacent open reading frames organized in one operon were localized by DNA sequencing. The functionality of the gene was demonstrated expressing the gene in *Escherichia coli* and using the purified gene product in a photometrical NADPH dependent test based on the substrate DXP generating system. While the content of one of the central intermediates of the isoprenoid biosynthesis (dimethylallyl diphosphate = DMADP) was significantly ($P \leq 0.001$) increased in *E. coli* cells overexpressing the DXP synthase gene (*dxs*) of *S. leopoliensis*, overexpression of *dxr* does not lead to an elevated DMADP level. Since even in strains harboring an expression fusion of *dxs* the additional overexpression of *dxr* does not influence the DMADP content, it is concluded that Dxs but not Dxr catalyzes a rate limiting step of the non-mevalonate isoprenoid biosynthesis.

Key words: 1-Deoxy-D-xylulose 5-phosphate synthase gene; *dxs*; 1-Deoxy-D-xylulose 5-phosphate; 1-Deoxy-D-xylulose 5-phosphate reductoisomerase gene; *dxr*; Isoprenoid biosynthesis

1. Introduction

Heterotrophic bacteria and plastids synthesize isoprenoids in a series of reactions totally different from the classical mevalonate pathway [1–3]. In the initial step of this pathway, glyceraldehyde 3-phosphate (GAP) and pyruvate are converted to 1-deoxy-D-xylulose 5-phosphate (DXP) catalyzed by the DXP synthase (Dxs). The activity was identified in bacteria and extracts of chloroplast containing plant tissues [2,3]. The gene encoding this enzyme (*dxs*) was identified and functionally analyzed for *Escherichia coli* and *Mentha x piperita* [4–6]. The next step in the bacterial and chloroplastic isoprenoid biosynthesis is the formation of 2-*C*-methyl-D-erythritol 4-phosphate (MEP) catalyzed by the DXP reductoisomerase (Dxr). The responsible gene was first isolated from *E. coli*, *Mentha* and *Arabidopsis* [7–10]. Recently, it was found that in the subsequent steps, a cytidyl group is transferred to MEP catalyzed by the *ygbP*-encoded gene product of *E. coli* [11]. The resulting 4-diphosphocytidyl-2-*C*-methylerythritol is phosphorylated by the product of the *E. coli ychB* gene [12] and in a third reaction 2-*C*-methylerythritol 2,4-cyclodiphosphate is formed by splitting off cytidyl monophosphate [13]. The following reactions of bacterial and plastidic isoprenoid biosynthesis that lead to isopentenyl diphosphate (IDP) and dimethylallyl diphosphate (DMADP), the general precursors of isoprenoids, are still unknown. As DXP is not only involved in isoprenoid biosynthesis but is also the precursor for thiamine and pyridoxol synthesis [4,14,15], the presence of a *dxs* gene is not indicative for the mevalonate independent pathway. Therefore the subsequent reaction, e.g. formation of MEP, and the presence of the *dxr* gene is a much better proof for the existence of this so called MEP pathway. Whereas the existence of this pathway in *Synechocystis* sp. PCC 6714 was concluded from ^{13}C-labeling studies [16,17], and the functional presence of the *dxs* gene was demonstrated for the cyanobacterium *Synechococcus leopoliensis* [17], the functionality of the gene *dxr* that is characteristic for the MEP pathway still remained to be shown. The present study aimed to establish the existence and the functionality of *dxr* for *S. leopoliensis* and to identify the rate limiting step in isoprenoid biosynthesis.

2. Materials and methods

2.1. Chemicals and enzymes

Antibiotics were supplied by Boehringer (Mannheim, Germany). All restriction enzymes, ligase and PCR-Taq-polymerase were delivered from Gibco BRL (Berlin, Germany). Fosmidomycin was kindly synthesized and provided by Prof. Dr. W. Boland and Dr. A. Jux (MPI of Chemical Ecology, Jena, Germany). Other chemicals were supplied by Merck (La Roche Diagnostics, Mannheim, Germany) or Roth (Karlsruhe, Germany).

2.2. Strains and plasmids

The cosmids of the gene library [17], derivatives of pCR2.1 (Invitrogen BV, Groningen, The Netherlands), pQE30, pQE50 (Qiagen, Hilden, Germany) and pGS72 [19] were amplified in *E. coli* TG1 [20].

2.3. Construction of a dxr gene probe

Oligonucleotides (Roth, Karlsruhe, Germany) were designed for conserved regions of known *dxr* gene sequences (EMBL: Q55663

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Synechocystis sp., P45568 *E. coli*): *dxr*-for: 5′-GTG GTC ACA GGT ATT GT(AGCT) GG(AGCT) TG(CT) GC(AGCT) GG-3′; *dxs*-rev: 5′-TGC ATG CAC GGA TAT TT(AG) TC(AG) TG(AG) TC(AGCT) GG-3′.

These oligonucleotides were used to amplify a segment of the *dxr* gene with genomic DNA from *S. leopoliensis* in a thermocycler (Personal cycler, Biometra, Göttingen, Germany) in 36 circles (1 min 94°C, 1 min 62°C, 1 min 72°C). The assay contained 2.5 U Taq-polymerase (Gibco BRL), 1×PCR buffer (Gibco BRL), 4.0 mM $MgCl_2$, 0.2 mM of each dNTP, 50 pmol of each oligonucleotide, 50 ng genomic DNA of *S. leopoliensis* in a final volume of 50 μl. The amplified product was cloned into pCR2.1 (Invitrogen BV, Groningen, The Netherlands) and was non-radioactively labeled by incorporation of digoxigenin-labeled nucleotides using the DIG Nucleic Acid Labeling and Detection kit (Boehringer, Mannheim, Germany).

2.4. Construction of expression fusions

For the expression fusion oligonucleotides (Roth) with restriction sites (*Sst*I or *Bam*HI at 5′-end, *Hin*dIII at 3′-end) were designed: *dxr-Sst*I-for: 5′-AAT TTC TGA **GAG CTC** CCC GTG AAA GCA GTG-3′; *dxr-Hin*dIII-rev: 5′-GAT AGA CC**A AGC TT**C TGC CCT AAA C-3′; *dxs-Bam*HI-for: 5′-GTT GCG CGT CTT G**GG ATC C**AC CGG AGG ACG TCT G-3′; *dxs-Hin*dIII-rev: 5′-CAA GCC GCA GTC **AAG CTT** GCG CTA CTC AAG C-3′.

PCR was performed in 36 cycles (1 min 94°C, 1 min 55°C, 3 min 72°C) in a 50 μl assay containing 1 U Taq-polymerase (Gibco BRL), 1×PCR buffer (Gibco BRL), 2.0 mM $MgCl_2$, 0.2 mM of each dNTP, 50 pmol of each oligonucleotide, 1 ng of plasmid DNA.

2.5. DNA sequencing

Overlapping restriction fragments of pCR005 were cloned in pCR2.1 and both strands of the 5.5 kbp *Hin*dIII–*Eco*RI insert (Fig. 2) were sequenced using cycle sequencing dideoxy chain termination reactions with Big Dye Terminators (PE Applied Biosystems, Weiterstadt, Germany) and the universal forward and backward primer (Gibco BRL) or sequence-specific oligonucleotides (Roth). The sequence was analyzed on an ABI PRISM-System 310 (PE Applied Biosystems). The EMBL accession number of the complete 5476 bp DNA sequence is AJ250721.

2.6. Purification of Dxs and Dxr from transformed E. coli TG1

To purify the gene products of *dxs* and *dxr*, the genes were cloned in frame into the plasmid pQE30 harboring a His coding region behind the *lac*-promotor (Qiagen, Hilden, Germany). 5 ml of overnight pre-cultures grown in LB at 37°C were inoculated into 250 ml LB and incubated for 1 h with vigorous shaking until an OD_{600} of 0.6 was reached. Expression was induced by adding 4 μM isopropyl β-D-thiogalactoside (IPTG, Gibco BRL), and the cultures were incubated for an additional 4–5 h. The cells were harvested by centrifugation at 4000×*g* for 20 min. The pellets were resuspended in lysis buffer (50 mM NaH_2PO_4 pH 8, 300 mM NaCl, 10 mM imidazole, 5 mM 2-mercaptoethanol (ME), 5 ml buffer per g wet weight) and disrupted twice with a French® pressure cell press (SLM Instruments, Inc., Urbana, USA) at 1400 bar and 0–4°C. After centrifugation (10 000×*g*, 20 min, 4°C), 4 ml of the lysate was mixed with 1 ml of 50% Ni-NTA slurry (QIAexpress Type IV kit, Qiagen) at 4°C for 1 h according to the batch purification protocol. The mixture was loaded into an empty column (Qiagen), washed twice with 4 ml washing buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, 5 mM ME) and eluted with 2 ml elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, 5 mM ME). All fractions were analyzed by SDS–PAGE on a pre-cast 4–20% gradient Tris–glycine gel (Novex, Frankfurt, Germany) at 125 V for 2 h. After 1 h of incubation in fixation solution (79 ml H_2O, 1 ml phosphoric acid 85%, 20 ml methanol), the gels were stained overnight with Roti blue (Roth, Karlsruhe, Germany), a colloidal Coomassie-staining solution, and washed with 25% methanol (Fig. 3). The gels were dried for 5 h at 40°C under vacuum.

2.7. Dxr enzyme assay

The eluted protein samples were applied to a PD-10 column (Pharmacia Biotech, Freiburg, Germany) preequilibrated with assay buffer (150 mM Tris–HCl pH 7, 5 mM $MgCl_2$, 5 mM ME, 1 mM thiamine diphosphate, 10% glycerol) and eluted with 3.5 ml assay buffer. Protein concentrations were determined by the Bradford assay [21]. To synthesize DXP, a preincubation was performed at 25°C for 16 h containing 50–100 μg/ml Dxs, 33 mM fructose 1,6-diphosphate, 66 mM neutralized pyruvate, 1 U fructose-aldolase, 50 U triosephosphate isomerase (Boehringer, Mannheim, Germany) in a final volume of 1 ml. 250 μl of this mixture was completed with the purified Dxr (3 μg/ml) and 1 mM $MnCl_2$. The reaction was initiated by adding NADPH or NADH to a final concentration of 0.125 mM. The oxidation of NADPH or NADH was monitored by a Lambda 2 UV/VIS Spectrometer (Perkin Elmer, Ueberlingen, Germany) at 340 nm during 15 min at 25°C.

2.8. Determination of DMADP in E. coli cells

To determine the amount of the cellular DMADP content, 5 ml of a pre-culture grown in LB medium at 37°C for 10 h was inoculated into 100 ml M9 medium [22] supplemented with 1 ml FeEDTA (final concentration 0.5 mM) and 10 ml $MnSO_4$ (0.66 mM end concentration) and incubated as described [18]. Determination of DMADP was performed by the detection of isoprene after acidic release of the diphosphate group of DMADP [23]. For this purpose, the cell pellets were resuspended in 500 μl of 0.5 M H_2SO_4 in gas-tight vials. The vials were heated at 70°C for 2 h. After removing the liquid phase with a gas-tight Hamilton syringe, the isoprene released from DMADP was assayed by gas chromatography and a flame ionization detector as described [24]. Significance was tested by the use of Student's *t*-test.

3. Results and discussion

3.1. Amplification of the dxr gene probe

As a first step towards the identification of the gene encoding Dxr, the characteristic enzyme for the MEP pathway, a suitable gene probe was developed. The deduced *dxr* sequence of *E. coli* (P45568) was therefore compared to the hypothetical Dxr-like protein sequence of the sequenced genome of *Synechocystis* sp. (Q55663) [25] to localize conserved regions. This allowed to design suitable oligonucleotides (*dxr*-for, *dxr*-rev; Section 2) enabling amplification of a 667 bp segment of *S. leopoliensis* SAUG 1402-1 DNA in a PCR. The deduced amino acid sequence of the segment shared 48.7% identity with the Dxr of *E. coli* and 80.6% with the peptide sequence of *Synechocystis* sp. In the next step, the amplified *dxr* segment was labeled with digoxigenin (Section 2.3) and was used as a gene probe against digested chromosomal DNA of *S. leopoliensis*. With all restriction enzymes used, distinct hybridization bands were visible (Fig. 1). Whereas *Eco*RI and *Hin*dIII digestion resulted in only one hybridization signal, *Sal*I and *Xho*I digestions led to two hybridization bands. Since the sequenced gene probe harbors a *Sal*I and a *Xho*I restriction site, but neither *Eco*RI nor *Hin*dIII sites, this observation confirmed that the gene probe indeed originated from the chromosomal DNA of *S. leopoliensis*.

3.2. Identification of dxr carrying cosmids in a genomic cosmid library of S. leopoliensis

A gene library of *Xho*I fragments of *S. leopoliensis* SAUG 1402-1 ligated into pVK100 [18] was screened using the digoxigenin-labeled *dxr* gene probe. Three of 400 screened cosmids showed positive hybridization signals. A restriction map of one of these clones (pAN005) carrying a 26.5 kbp insert of the *S. leopoliensis* genome was established (Fig. 2). Because of identical hybridization patterns of the cosmid and the chromosomal DNA (Fig. 1), it was concluded that the identified cosmid pAN005 indeed contained an original segment of the *S. leopoliensis* genome. The region responsible for the hybridization signal was localized on a 5.5 kbp *Hin*dIII–*Eco*RI segment of the cosmid pAN005 (Fig. 2) by additional hybridization experiments (data not shown).

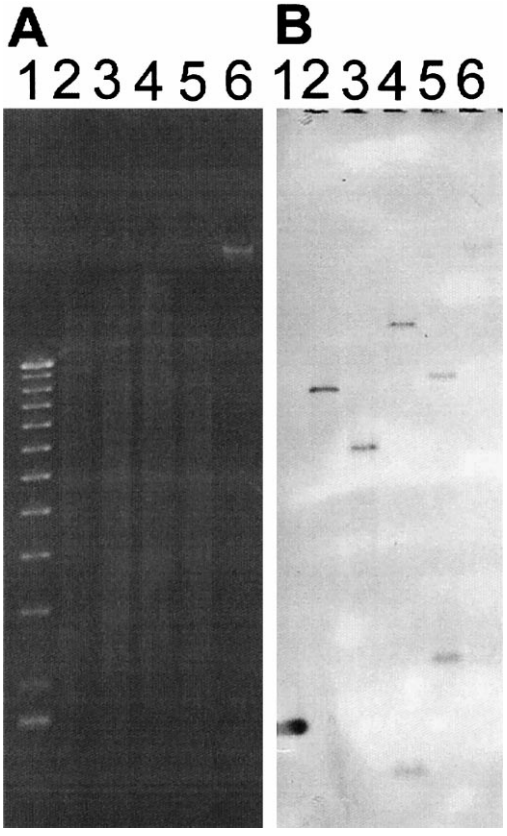

Fig. 1. Hybridization of a *dxr* segment of *S. leopoliensis* to digested genomic DNA of *S. leopoliensis*. (A) Agarose gel of digested and undigested genomic DNA. (B) Southern blot hybridized with a 667 bp amplified segment of the *dxr* gene. Lanes: 1 = 1 kbp ladder (Gibco BRL, Berlin, Germany), 2 = *Eco*RI, 3 = *Hin*dIII, 4 = *Sal*I, 5 = *Xho*I, 6 = undigested genomic DNA.

3.3. Sequencing of the locus and identification of six open reading frames (ORFs)

After subcloning the 5.5 kbp *Hin*dIII–*Eco*RI fragment into pCR2.1, the segment was sequenced on both strains using subclones and specific oligonucleotides. Three adjacent ORFs (ORF1, ORF2 and ORF3), putatively belonging to one operon, were identified from position 999 to 3757 of the total sequence. ORF1 starts with a GTG at position 999 and stops with a TAG at position 2207 encoding a protein of 402 amino acids. Nine bases after the stop codon of the ORF1, ORF2 starts with a GTG at position 2217 and stops at position 2816 with TAG. After 29 bases, the start codon of ORF3 is localized (position 2846). This reading frame encodes a protein of 304 amino acids and stops at position 3757 with TAA. The use of the rare start codon GTG has also been described for *hypA* (EMBL no. X97797) and ORF2 of the *dxs* operon (EMBL no. Y18874) of *S. leopoliensis*. Coding in the opposite direction, ORF4 stops at position 4410 with TGA. ORF5 starts at position 4384 with ATG and stops at position 3791 with TGA. Upstream of ORF5, ORF6 starts at position 856 with ATG. The start of ORF4 and the stop of ORF6 are outside of the sequenced 5.5 kbp *Hin*dIII–*Eco*RI fragment. All ORFs (except ORF6) were preceded by putative ribosome binding sites [26] 6–14 bp upstream of the start codons (ORF1: GAGGG, ORF2: AGGG, ORF3: AAGG, ORF5: GGGAA).

3.4. Homology of the deduced ORF1 to Dxr

The deduced amino acid sequence of ORF1 showed highest similarity (71.1% identity) to the putative Dxr (SLL0019) of the cyanobacterium *Synechocystis* sp. (strain PCC 6803). Especially the putative NADPH binding motif 'LGSTGSIG' [7] was completely conserved at position 7 of the deduced amino acid sequence. In contrast to the situation in *S. leopoliensis*, where the gene *dxr* is part of an operon, in *Synechocystis* sp. [25] the gene is monocistronic. A significantly higher identity (63.4%) was detected to the eukaryotic Dxr of *Arabidopsis thaliana* [10] than to the Dxr of *E. coli* [7] with only 42.3% identical residues, which confirms the close relationship between cyanobacteria and chloroplasts. Because of its high homologies, ORF1 was named *dxr*.

3.5. Homology of the deduced ORF2 to a protein of the pseudouridine synthase family

The deduced ORF2 peptide sequence showed identities to the hypothetical protein SLR0612 of the *Synechocystis* sp. genome [25] and to the protein YmfC of *E. coli* [27] both belonging to the family 1 of pseudouridine synthases (54.7% and 51.3% identity, respectively). In contrast to *S. leopoliensis* neither in *Synechocystis* sp., nor in *E. coli* nor in *Bacillus subtilis*, the gene encoding this protein is organized in an operon together with the *dxr*. No homology to any other actually known gene of the MEP pathway was found [11–13].

3.6. Homology of the deduced ORF3 to a hypothetical protein in Synechocystis sp.

77.9% similarity of the deduced ORF3 amino acid sequence was found to a hypothetical 73.7 kDa protein SLL1033 of *Synechocystis* sp. (P72756) [25]. Slightly lower similarities were detected to a putative regulatory protein of *Streptomyces coelicolor* (CAB90888) and to a putative protein phosphatase of *Chlamydia* (AAF39372) with 63.7% and 59.7%, respectively. No homology was found to the identified other genes of the MEP pathway [11–13]. ORF1, ORF2 and ORF3 putatively belong to one operon, which is not located next to the *dxs* operon [18], although the Dxr and the Dxs are catalyzing subsequent steps in the isoprenoid pathway. Also

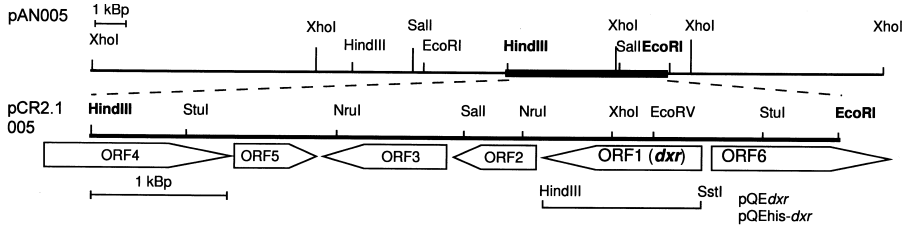

Fig. 2. Physical map of the *dxr* carrying 26.5 kbp insert of the cosmid pAN005, the subcloned 5.5 kbp insert of pCR005 with the localized ORFs and the expression fusions in pQE50 and pQE30.

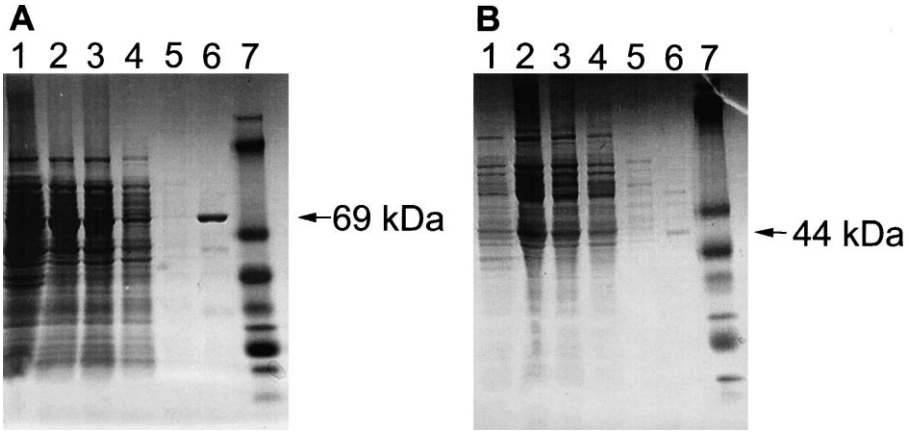

Fig. 3. Purification of Dxs (A) and Dxr (B) analyzed by SDS–PAGE. Lanes: 1 = crude extracts of cells induced with IPTG, 2 = cleared lysate, 3 = flow-through of the lysate on the Ni-NTA agarose column, 4 = first washing step of the Ni-NTA agarose column, 5 = second washing step, 6 = eluates of Dxs (A) and Dxr (B), 7 = multimark multi-colored standard (Novex, Frankfurt, Germany). Equal partitions of each fraction were loaded on the gel corresponding to 30 μg protein of crude extract.

in other prokaryotic organisms like *E. coli*, *B. subtilis* and *Synechocystis* sp. the *dxs* is not located in the neighborhood of the *dxr*.

3.7. Homology of the deduced ORF4, ORF5 and ORF6

ORF4, ORF5 and ORF6 are coding complementarily to the operon. Highest similarity (74.3%) of the deduced ORF4 polypeptide sequence was found to the hypothetical 52.6 kDa protein SLR1285 of *Synechocystis* sp. [25] and a lower but significant similarity to a group of prokaryotic histidine kinases of *Streptococcus pneumoniae* (Q9X4S9), *Pseudomonas aeruginosa* (O34206) and *B. subtilis* (P23545), indicating that ORF4 encodes also a histidine kinase. The deduced amino acid sequence of ORF5 showed 62.4% similarity to the phosphoglycerate mutase of the bacteria *Deinococcus radiodurans* (Q9RUJ3) and a surprisingly lower similarity of 57.9% to a hypothetical protein SLR1748 of the cyanobacteria *Synechocystis* sp. [25]. The highest identity (69.1%) of the deduced ORF6 peptide sequence was found to the hypothetical protein SLR0940 of *Synechocystis* sp. [25] with similarity to a ζ-carotene desaturase precursor of *A. thaliana* (Q9SS13), an enzyme in the late isoprenoid biosynthesis. Whereas *A. thaliana* harbors a chloroplastidic leader sequence at the N-terminus, this precursor sequence is missing in the two cyanobacteria which was expected as the enzymes in the cyanobacteria do not have to cross a chloroplast envelope to reach their destination. Therefore the gene of *S. leopoliensis* probably encodes a ζ-carotene desaturase.

3.8. Construction of expression fusions

In order to test the functionality of the *dxr* gene of *S. leopoliensis*, the substrate DXP was needed. As DXP is not commercially available, it was aimed to synthesize DXP using the functional *dxs* gene from *S. leopoliensis* [18]. To construct an expression fusion of *dxs*, a *Bam*HI and a *Hin*dIII site were introduced at the 5′-end and the 3′-end, respectively, via PCR using the oligonucleotides *dxs*-*Bam*HI-for and *dxs*-*Hin*dIII-rev (Section 2). Analogous, an *Sst*I and a *Hin*dIII site were introduced in the *dxr* gene. This allowed the cloning of the *dxr* gene in frame behind the promotor–operator element of the pQE50 and pQE30 (Fig. 2). All constructions were verified by DNA sequencing.

3.9. Purification of gene products of dxs and dxr

The enzymes Dxr and Dxs were purified from *E. coli* carrying pQEhis-*dxs* or pQEhis-*dxr*, respectively. The vector pQE30 was used because of the six His coding region in front of the genes, which enables the reversible interaction with Ni-NTA agarose for purification. As both proteins were found to be partially soluble, a batch purification under native conditions was performed. 3.0 μg of purified Dxs protein was obtained from 0.75 g wet weight of TG1(pQEhis-*dxs*) (Fig. 3A). The band of the overexpressed Dxs was already visible in high amounts in the crude cell extract and in the cleared lysate. After two washing steps, the purified Dxs gave a prominent band on SDS–PAGE at the expected size of 69 kDa. 0.5 μg purified Dxr protein was obtained from 1.0 g wet weight of TG1(pQEhis-*dxr*) (Fig. 3B). Analyzing the eluates on a SDS–PAGE, two bands were detected, but the smaller and more intense band had the expected size of 44 kDa deduced from the amino acid sequence. The yield of purified Dxr was less than for the Dxs because the Dxr protein was less soluble and the major protein content was found in the pellet (data not shown).

3.10. Functional analysis of the purified Dxr

The functionality test was divided into two parts. The first part was the in vitro production of DXP by the purified Dxs protein. The enzyme Dxs synthesizes DXP using the substrates pyruvate and GAP as described [4,5]. The use of GAP was avoided because of its instability at room temperature. Instead of this, GAP was produced by the aldolase and triosephosphate isomerase from fructose 1,6-bisphosphate [4]. The produced GAP was metabolized with pyruvate to DXP catalyzed by the purified Dxs in an overnight reaction. DXP is the known substrate of Dxr which can be converted to MEP by an NADPH dependent reduction [7]. Therefore an enzyme assay was designed monitoring the oxidation of NAD(P)H photometrically (Table 1). A linear NADPH oxidation was observed for 15 min in the complete assay in the presence of Dxr and the substrates of the reactions. If one of the enzymes (Dxs, Dxr) or one of the substrates was missing in the assay, the NADPH oxidation rate was less than 7.5% of the complete assay (Table 1). Thus, the activity of Dxr was significantly higher than the background NADPH oxidation

Table 1
Dxr dependent NADPH or NADH oxidation

Enzyme assay	Consumption of NAD(P)H (pmol/s)	Consumption of NAD(P)H (nmol/s/mg protein)
Complete assay	23.7 ± 6.2	4.0 ± 1.1
Complete assay with NADH[a]	3.8 ± 1.4	0.6 ± 0.2
7.5 μM fosmidomycin	12.3 ± 0.3	2.1 ± 0.1
15 μM fosmidomycin	7.6 ± 0.8	1.3 ± 0.1
30 μM fosmidomycin	5.8 ± 0.4	1.0 ± 0.1
60 μM fosmidomycin	1.5 ± 1.1	0.3 ± 0.2
Without Dxr	0.3 ± 1.6	not determinable
Denatured Dxr	0.0 ± 0.3	0.0 ± 0.1
Without Dxs	0.4 ± 0.7	0.1 ± 0.1
Denatured Dxs	1.2 ± 1.7	0.2 ± 0.3
Without pyruvate	1.9 ± 0.7	0.3 ± 0.1
Without fructose 1,6-bisphosphate	0.8 ± 0.9	0.0 ± 0.2
Without pyruvate and fructose 1,6-bisphosphate	0.9 ± 0.5	0.2 ± 0.1
Without overnight synthesized DXP	1.3 ± 0.8	0.2 ± 0.1

The complete enzyme assay containing 50–100 μg/ml Dxs, 33 mM fructose 1,6-bisphosphate, 66 mM pyruvate, 1 U fructose-aldolase, 50 U triosephosphate isomerase for the overnight production of DXP was completed with Dxr (3 μg/ml) and 1 mM $MnCl_2$ after preincubation. The reaction was initiated by adding NADPH or NADH (final concentration 0.125 mM) and monitored photometrically at 340 nm during 15 min at 25°C.
[a]Except this enzyme assay, all assays were performed with NADPH. At least three independent repetitions were performed for each assay.

rate and the purified Dxr of *S. leopoliensis* was indeed functional. NADH instead of NADPH as electron donor led to a six times lower oxidation rate, indicating that the enzyme Dxr prefers NADPH as a cosubstrate but has a higher reaction velocity with NADH than the enzyme of *E. coli* where only 1% NADH dependent enzyme activity was detected compared to the NADPH dependent activity [7]. Fosmidomycin, a structure analog of DXP, is known as a specific inhibitor of the Dxr in bacteria [28]. In order to test that Dxr of *S. leopoliensis* can also be inhibited by fosmidomycin and to show that the NADPH oxidation was indeed due to a Dxr-catalyzed reaction, fosmidomycin was added in several concentrations. While 7.5 μM fosmidomycin reduced the Dxr activity to 50%, 60 μM fosmidomycin almost completely inhibited the reaction (Table 1). As the expressed enzyme (i) was found to reduce NADPH in the presence of DXP, (ii) could be inhibited by fosmidomycin and (iii) had an amino acid sequence with high similarity to other known Dxr polypeptides (see Section 3.4), it was concluded that the enzyme indeed represents the Dxr of *S. leopoliensis*. As the enzyme Dxr is indicative for the non-mevalonate isoprenoid biosynthesis, the existence and the functionality of the Dxr in *S. leopoliensis* establish the existence of the mevalonate independent isoprenoid pathway via MEP in cyanobacteria.

3.11. Influence of the dxs and dxr expression fusions on cellular DMADP content

DMADP and the isomeric IDP are central intermediates of isoprenoid biosynthesis, and all isoprenoids in the cell derive from these molecules. To identify limiting steps in the biosynthesis of DMADP and to study the functionality of identified *dxr* in vivo, the DMADP content of the cells was monitored in dependency of the presence of overexpressed *dxs* and/or *dxr* of *S. leopoliensis*. For this purpose, *E. coli* cells were transformed with pQE*dxr* and with pQE*dxs*. The transconjugants with the *dxs* insert showed a significant ($P \leq 0.001$) eight times increased cellular DMADP level of 2.2 pmol/mg cells (Table 2), compared to wild type *E. coli* TG1 or the transconjugants with the insertless vector pQE50, or even the strain harboring pQE*dxr* (0.2–0.3 pmol/mg). Thus, pQE*dxs* but not pQE*dxr* stimulated the DMADP production in *E. coli*, indicating that the Dxs-catalyzed reaction is one of the limiting steps of DMADP production in wild type *E. coli*. Therefore it appears that in the pQE*dxr* carrying strain, the amount of DXP was too low to enable increased DMADP production. To increase the internal DXP level, it was reasonable to combine *dxs* and *dxr* in one cell. For this purpose, *dxs* was cloned into a vector of a different incompatibility group. Therefore the *lac*-promotor and the *dxs* carrying region of pQE*dxs* (ColE1 group) were cloned into the Km resistance gene of pGS72 (IncP group) resulting in the still Tc resistant *dxs* carrying vector pGS*dxs*. The strain carrying pGS*dxs* had a two times higher DMADP content than the strain with the insertless plasmid, indicating that *dxs* is also functional in the pGS72 vector. Because of the changed growth pattern due to the second antibiotic, the transconjugant with both insertless vectors showed a slightly higher cellular DMADP content of 0.4 pmol/mg cells compared to the control clone harboring only one of the plasmids. When *E. coli* was transformed with both vectors, the level of the DMADP concentration depended on whether the *dxs* gene was ligated into the low copy (pGS72) or into the high copy vector (pQE50). The DMADP content of TG1(pGS*dxs*/pQE50) with *dxs* expressed in the low copy vector was detected at 2.3 pmol/mg whereas the DMADP content of TG1(pGS72/pQE*dxs*) with *dxs* expressed in a

Table 2
DMADP content of transconjugant *E. coli* TG1 cells

E. coli clones	DMADP content (pmol/mg cells)
TG1	0.3 ± 0.1
TG1(pQE50)	0.3 ± 0.1
TG1(pQE*dxs*)	2.2 ± 0.9
TG1(pQE*dxr*)	0.2 ± 0.1
TG1(pGS72)	0.4 ± 0.2
TG1(pGS*dxs*)	1.0 ± 0.2
TG1(pGS72/pQE50)	0.4 ± 0.1
TG1(pGS*dxs*/pQE50)	2.3 ± 1.5
TG1(pGS72/pQE*dxs*)	3.1 ± 1.1
TG1(pGS*dxs*/pQE*dxr*)	2.5 ± 1.2

The DMADP content was determined gas chromatographically after acid hydrolysis into isoprene in 50 mg cell pellets of overnight liquid cultures grown in minimal glucose containing M9 medium [22] supplemented with 1 mM pyruvate. The experiments were performed at least with six independent repetitions.

high copy number plasmid was at 3.1 pmol/mg. There was no significant difference ($P \leq 0.77$) in the DMADP content between cells harboring pGS*dxs*/pQE50 (2.3 pmol/mg cells) and pGS*dxs*/pQE*dxr* (2.5 pmol/mg cells). This observation indicated that the Dxs is one of the limiting enzymes in the biosynthesis of isoprenoids in bacteria and that overexpression of Dxr does not cause an additional production of DMADP even when the cells had an elevated DXP level. The observed regulatory role of the Dxs amount in the isoprenoid biosynthesis is in accordance with the observation that in ripening tomatoes, where high amounts of carotenoids are produced, the amount of *dxs* mRNA is increased [29].

3.12. Concluding remarks

In the present work, it was shown that cyanobacteria like *S. leopoliensis* possess a functional *dxr* gene. As this enzyme catalyzes the formation of MEP which is indicative for the presence of the mevalonate independent pathway of isoprenoid biosynthesis, it is strongly established that cyanobacteria synthesize isoprenoids via the MEP pathway. This supports the theory that the MEP pathway entered the plant cell via a cyanobacterial-like chloroplast ancestor according to the endosymbiont theory [3,16]. Other authors question this theory [30] as they could not detect any activity of an IDP isomerase in cyanobacteria which is normally present in plants and catalyzes the reversible conversion of IDP to DMADP. However, also for *E. coli* it was found that an isomerase is not necessary for the survival of the cells [31,32] because DMADP and IDP can be synthesized separately by the MEP pathway in *E. coli* [33] as apparently in *Synechocystis* [30] too. The absence of the isomerase in cyanobacteria does not infer with a cyanobacterial-like chloroplast ancestor, as this enzyme could have been derived from the cytoplasmical IDP isomerase which was probably already present from the mevalonate pathway of isoprenoid biosynthesis of the eukaryotic cell which took up the chloroplast ancestor. Thus the IDP isomerase found in plant chloroplasts [23] can derive from the cytoplasmic mevalonate pathway rather than from the plastid ancestor with its MEP pathway. To efficiently produce isoprenoids in *E. coli*, e.g. for biotechnological purpose, it can be concluded from the expression studies of *dxs* and/or *dxr* in *E. coli* that it is sufficient to overexpress *dxs* rather than *dxr* to obtain an elevated DMADP level and a more efficient turnover in this pathway.

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