A *Synechococcus leopoliensis* SAUG 1402-1 operon harboring the 1-deoxyxylulose 5-phosphate synthase gene and two additional open reading frames is functionally involved in the dimethylallyl diphosphate synthesis

Barbara Miller, Thomas Heuser, Wolfgang Zimmer*

Fraunhofer Institut für Atmosphärische Umweltforschung, Kreuzeckbahnstr. 19, D-82467 Garmisch-Partenkirchen, Germany

Received 19 August 1999; received in revised form 4 October 1999

Abstract Experiments have been performed to prove the existence and the functionality of the novel mevalonate independent 1-deoxyxylulose 5-phosphate isoprenoid biosynthesis pathway in cyanobacteria. For this purpose, a segment of the 1-deoxyxylulose 5-phosphate synthase gene (*dxs*) was amplified from *Synechococcus leopoliensis* SAUG 1402-1 DNA via PCR using oligonucleotides for conserved regions of *dxs*. Subsequent hybridization screening of a genomic cosmid library of *S. leopoliensis* with this segment has led to the identification of an 18.7 kbp segment of the *S. leopoliensis* genome on which a *dxs* homologous gene and two adjacent open reading frames organized in one operon could be localized by DNA sequencing. The three genes of the operon were separately expressed in *Escherichia coli*, proving that the identified cyanobacterial *dxs* is functionally involved in the formation of dimethylallyl diphosphate, one basic intermediate of isoprenoid biosynthesis.

Key words: 1-Deoxyxylulose 5-phosphate synthase gene; Dimethylallyl diphosphate; Isopentenyl diphosphate; Isoprenoid biosynthesis; *dxs*

1. Introduction

Recently, it has been found that heterotrophic bacteria synthesize isoprenoids following a pathway totally different from the classical mevalonate pathway [1,2]. In the novel pathway, glyceraldehyde 3-phosphate and pyruvate are the substrates for an initial transketolase reaction resulting in 1-deoxyxylulose 5-phosphate [2]. This reaction was also identified in plants and green algae and was found to occur mainly exclusively in the chloroplasts. In contrast, the isoprenoids in the cytoplasm of plant cells are synthesized via the classical mevalonate pathway [3–8]. The genes responsible for the 1-deoxyxylulose 5-phosphate synthase (Dxs) were first sequenced and functionally analyzed for *Escherichia coli* and for *Mentha piperita* [9,10]. 1-Deoxyxylulose 5-phosphate is not only the precursor for chloroplastic and bacterial isoprenoid biosynthesis but is also involved in thiamine and pyridoxol synthesis [11,12]. Also a gene from *E. coli* was isolated, encoding an enzyme which is functionally involved in the subsequent first reduction and isomerization step of 1-deoxyxylulose 5-phosphate [13,14]. This gene could subsequently be identified in *Mentha* and *Arabidopsis* [15,16]. However, no other genes for enzymes catalyzing the further reductions and phosphorylation to form dimethylallyl diphosphate (DMADP) or isopentenyl diphosphate, the common precursors of all isoprenoids, are known. Possibly, the alternative pathway entered the plant via the green bacterial chloroplast ancestor according to the endosymbiont theory. Therefore, also the blue-green cyanobacteria commonly accepted as chloroplast-related organisms should synthesize their isoprenoids via this mevalonate independent pathway. The existence of this pathway in *Synechocystis* sp. PCC 6714 was concluded from ^{13}C-labelling studies [17]. Therefore, it was intended in the present work to isolate genes involved in this novel pathway from the unicellular cyanobacterium *Synechococcus leopoliensis* (= *Anacystis nidulans*) and to prove the functional involvement of these genes in the biosynthesis of DMADP.

2. Materials and methods

2.1. Strains and plasmids

Derivatives of pUC18 (Gibco BRL, Berlin, Germany), pCRII (Invitrogen Corporation, San Diego, CA, USA), pQE50 (Qiagen, Hilden, Germany) and the cosmids of the gene library were amplified in *E. coli* TG1 [18].

2.2. Oligonucleotides and polymerase chain reaction (PCR)

Synthesized oligonucleotides (Roth, Karlsruhe, Germany) were used for the PCRs and for cycle sequencing in a thermocycler (Personal cycler, Biometra, Göttingen, Germany). The designed oligonucleotides for the amplification of a segment of the *dxs* gene and for the expression fusions of the genes in pUC18 and pQE50 were

1for: 5′-GTG ATC TGG GAT GTG GGA CAC CAG GCG TAT CC-3′
1rev: 3′-CGA CCA TAG CAC CCC CGC CTA CCC GGG TGG-5′
2for: 5′-G TTG CGC GTC TT**G AAT TC**C ACC GGA GGA CGT CT-3′
2rev: 3′-CGA ACT CAT CGC G**TT CGA A**CT GAC GCC GAA G-5′
3for: 5′-AGC GCG ATC GTG **GGA TCC** GCT TCT GAG ACC-3′
3rev: 3′-GTA CAG CGG AT**T TCG AA**C CGA CTT CTA ACG-5′
4for: 5′-GCC AAG TCA TG**G AGC TC**A AAC CTT GGC TGA AG-3′
4rev: 3′-GAT CTG AAC CGA AC**T TCG AA**G TAG TGG ACG-5′

These oligonucleotides were used in a PCR reaction with 36 circles (60 s denaturation at 94°C, 60 s annealing at 58°C, 90 s polymerization at 72°C) in a volume of 50 μl containing Taq-polymerase (Gibco BRL), 2 U, 10×Taq-polymerase buffer (Gibco BRL), 0.25 μmol $MgCl_2$, 50 pmol of each oligonucleotide, 50 ng genomic DNA of *S. leopoliensis* or DNA of pCR227.

*Corresponding author. Fax: (49) (8821) 73573.
E-mail: zimmer@ifu.fhg.de

PII: S0014-5793(99)01397-6

2.3. Construction of a genomic library of S. leopoliensis SAUG 1402-1

Isolated genomic DNA of *S. leopoliensis* was partially digested by *Xho*I and ligated into a *Xho*I-digested broad host range cosmid pVK100 [19]. These cosmids were in vitro packaged into lambda particles (Gigapack III Gold Packaging Extract, Stratagene, Amsterdam, The Netherlands) and were used to infect *E. coli* TG1 [18]. 1384 Tc^R Km^S separate clones were obtained harboring mainly 12–30 kbp segments of the *S. leopoliensis* genome.

2.4. DNA sequencing

Overlapping restriction fragments of pCR227 were cloned in pCRII or pUC18 and cycle sequencing dideoxy chain termination reactions with Big Dye Terminators (PE Applied Biosystems, Weiterstadt, Germany) and the universal forward primer (Gibco BRL), the universal backward primer (Gibco BRL) or sequence specific oligonucleotides were performed for both strands (see Fig. 2). The sequence was analyzed on an ABI PRISM-System 310 (PE Applied Biosystems). The EMBL, GenBank and DDBJ accession number of the complete 5802 bp DNA sequence is Y18874.

2.5. Determination of DMADP in E. coli cells

To measure the amount of DMADP in bacterial cells, 5 ml of an overnight pre-culture grown in LB medium was inoculated into 100 ml M9 medium [20] and incubated for 16 h at 37°C. After this period, 50 ml fresh M9 medium and neutralized pyruvate (final concentration 1 mM) were added and incubation was continued for 3 h. Pyruvate was added, as it can quickly enter the cells and thus increase one of the substrate concentrations of the Dxs. Cells were sedimented at $10\,000 \times g$ and washed once with 50 mM $CaCl_2$. Fifty mg of the pellet was filled into a 2 ml gas-tight, clear crimp seal vial (Supelco, Bellefonte, USA) and the vial was sealed immediately. Determination of DMADP was performed by the detection of isoprene after acidic release of the diphosphate group of DMADP [21]. For this purpose, the pellet in the vials was acidified by addition of 500 μl of 0.5 M H_2SO_4. The vial was then heated at 70°C for 2 h and the isoprene released from DMADP was assayed as described [22].

3. Results and discussion

3.1. Amplification of a segment of the dxs gene

In order to identify and analyze the *dxs* gene from cyanobacteria, analysis of conserved regions in the *dxs* gene of *E. coli* [9] and related sequences of hypothetical proteins in *Bacillus subtilis* and *Synechocystis* sp. PCC 6803 was performed. This led us to design suitable oligonucleotides (1for, 1rev; see Section 2) to amplify a 1085 bp segment of *S. leopoliensis* SAUG 1402-1 DNA in a PCR reaction. The deduced amino acid sequence of the sequenced segment shared 51.3% identity with the Dxs of *E. coli* [9]. It was proven by hybridization that the amplified segment originated from the chromosomal DNA of *S. leopoliensis* (Fig. 1). Positive hybridization signals were also detected when chromosomal DNA of *Anabaena variabilis* ATCC 29413 was used, indicating that the gene is also present in filamentous cyanobacteria.

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

The amplified *dxs* segment was labelled with digoxigenin and used to screen a gene library of *Xho*I fragments of *S. leopoliensis* SAUG 1402-1 in pVK100. Fifteen of 1384 screened clones showed positive hybridization signals. A restriction map of one of these cosmids, pAN227, carrying an 18.7 kbp insert of the *S. leopoliensis* genome was established (Fig. 2). The hybridization pattern for *Eco*RI, *Hin*dIII and *Xho*I corresponded with the hybridization pattern of the chromosomal DNA (Fig. 1). Because of these identical hybridization patterns, it was concluded that the identified cosmid

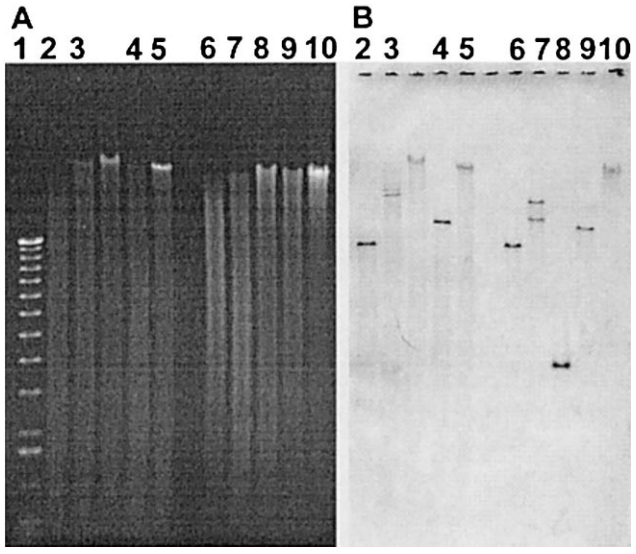

Fig. 1. Hybridization of a *dxs* segment of *S. leopoliensis* to digested genomic DNA of *S. leopoliensis* and *A. variabilis*. (A) Agarose gel of digested and undigested genomic DNA of *S. leopoliensis* and *A. variabilis*. (B) Southern blot hybridized with a 1.1 kbp amplified segment of the *dxs* gene of *S. leopoliensis*. Lanes: 1 = 1 kbp ladder (Gibco BRL, Berlin, Germany), 2 = *Eco*RI digest of *S. leopoliensis* DNA, 3 = *Hin*dIII digest of *S. leopoliensis* DNA, 4 = *Xho*I digest of *S. leopoliensis* DNA, 5 = undigested genomic DNA of *S. leopoliensis*, 6 = *Eco*RI digest of *A. variabilis* DNA, 7 = *Hin*dIII digest of *A. variabilis* DNA, 8 = *Sal*I digest of *A. variabilis* DNA, 9 = *Xho*I digest of *A. variabilis* DNA, 10 = undigested genomic DNA of *A. variabilis*.

pAN227 indeed represented an original segment of the *S. leopoliensis* genome. The region responsible for the hybridization signal could be localized on a 5.8 kbp *Eco*RI/*Hin*dIII segment of cosmid pAN227 (Fig. 2) by additional hybridization experiments (not shown).

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

After subcloning the 5.8 kbp *Eco*RI/*Hin*dIII segment into pCRII (pCR227), the segment was sequenced using subclones and specific oligonucleotides (Fig. 2). Three adjacent ORFs (ORF1, ORF2 and ORF3) were identified from position 1576 to 5439 of the total sequence. ORF1 with an ATG start codon at position 1576 and a TGA stop codon at position 3484 encoded a 633 amino acid protein. Eleven bases after the stop codon of ORF1, ORF2 starts with a GTG at position 3498 and stops at position 4749 with a TAA. The usage of the seldom start codon GTG has also been described for *hypA* of *S. leopoliensis* (EMBL no. X97797). Eight bp overlapping to ORF2 at position 4741, ORF3 starts with an ATG and stops at position 5437 with a TGA. As there is only a 11 bp gap in between ORF1 and ORF2 and an 8 bp overlap between ORF2 and ORF3, the three ORFs putatively belong to one operon. 772 bp upstream of the operon at position 804, another ORF (ORF4) starts in the opposite direction. In the 772 bp region between ORF4 and the operon as well as in the sequenced 363 bp after the operon, no other coding regions were detected. All ORFs were preceded by putative ribosome binding sites [23] 11–19 bp upstream of the start codons (ORF1, GGAGGA; ORF2, GAG; ORF3, GGAGG; ORF4, GAAGAG).

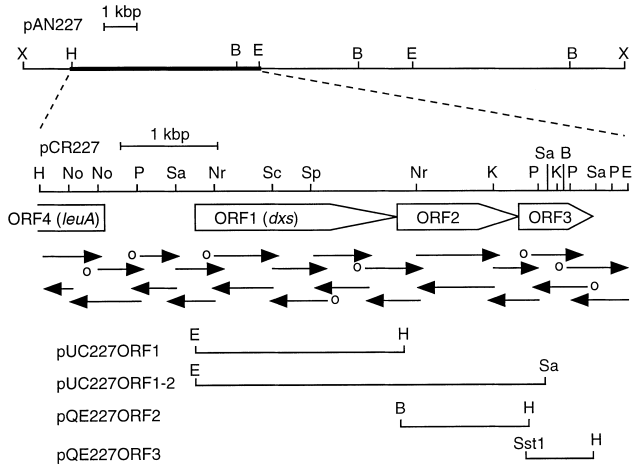

Fig. 2. Physical map of the 18.7 kbp insert pAN227, the subcloned 5.8 kbp insert of pCR227 with the localized ORFs and the expression constructions in pUC18 and pQE50. Below the map of pAN227, identified ORFs are indicated by open arrows and the sequencing strategy is given by black arrows. An 'o' indicates sequencing reactions started by a sequence specific oligonucleotide. The given restriction enzymes are: B = *Bam*HI, E = *Eco*RI, H = *Hin*dIII, K = *Kpn*I, Nr = *Nru*I, No = *Not*I, P = *Pvu*II, Sa = *Sal*I, Sc = *Sca*I, Sp = *Sph*I, X = *Xho*I.

3.4. Homology of the deduced ORF1 to Dxs

The deduced amino acid sequence of ORF1 showed 45.6% identical residues to the Dxs of *E. coli* [9] (Fig. 3). The highest similarity (74.7% identity) was detected to a hypothetical protein (sll1945) of the cyanobacterium *Synechocystis* sp. which, however, is not organized in an operon as found for the *S. leopoliensis* gene [24]. The deduced amino acid sequence of ORF1 showed similarity (46.0% identity) to the eukaryotic Dxs of *M. piperita* [10] as high as to the *E. coli* sequence, indicating the close relationship between cyanobacteria and chloroplasts. The consensus thiamine pyrophosphate binding motive was conserved in all of the sequences [25]. Analogous to these highly homologous sequences, ORF1 was designed *dxs*.

3.5. Homology of the deduced ORF2 to a hypothetical protein in Synechocystis sp.

The deduced ORF2 peptide sequence showed the highest similarity (38.0% identity) to the hypothetical protein slr1998 of the *Synechocystis* genome [24] which is located far away from the putative *dxs* single gene operon of this organism (Fig. 4). Next, a weaker similarity (21.1%) was detected to the *chlP* gene product of *Synechocystis* sp. PCC 6803, which catalyzes the stepwise reduction of geranylgeraniol to phytol during chlorophyll a biosynthesis [26]. Because of this homology and because of the location in an operon just behind the *dxs*, the ORF2 gene product of *Synechococcus* is possibly also a reductase involved in isoprenoid biosynthesis. However, ORF2 of *Synechococcus* had no homology to ORF3 of *E. coli* (Fig. 4), which was found in an operon just behind the *dxs* of *E. coli* [9] and was also supposed to be a reductase in the isoprenoid biosynthesis as it showed homology to the aldo-keto reductase superfamily [27]. No homology was found between ORF2 of *Synechococcus* and the gene located behind the putative *dxs* of *B. subtilis* (Fig. 4). In addition, no homology was detected to the 1-deoxyxylulose 5-phosphate reductoisomerase of *E. coli*, catalyzing the next step in the isoprenoid biosynthesis after the Dxs in bacteria [13].

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

Similarity (48.7%) of the deduced ORF3 amino acid sequence was found to a hypothetical protein slr1679 of *Synechocystis* sp. PCC 6803 [24]. However, in *Synechocystis*, the corresponding gene is located far away from the *dxs*-like gene and also far away from the above mentioned locus of sll1998, which is a significant difference in the organization of these genes in the cyanobacteria *Synechocystis* and *S. leopoliensis*, where the *dxs*, ORF2 and ORF3 are organized in one operon. ORF3 does not show similarity to any of the genes found in the *dxs* operon (Fig. 4) of *E. coli* [9].

3.7. Homology of the deduced ORF4 and leuA gene products

The highest similarity (69.8%) of the deduced ORF4 polypeptide sequence was found to the putative *leuA* gene product of *Synechocystis* sp. PCC 6803. *leuA* encodes the 2-isopropylmalate synthase which catalyzes the first step in the leucine biosynthesis. As the deduced ORF3 sequence also showed significant similarity to *leuA* gene products of other bacteria such as *E. coli* and *B. subtilis*, the gene from *S. leopoliensis* was designed *leuA*.

3.8. Construction of expression fusions of the identified dxs operon

In order to prove the functional involvement of the identified *dxs* operon of *S. leopoliensis* in the biosynthesis of isoprenoids, an *Eco*RI, a *Bam*HI or a *Sst*I site was introduced at the beginning of ORF1, ORF2 and ORF3 and a *Hin*dIII site

Table 1
DMADP content of transconjugant *E. coli* strains

E. coli strain	$OD_{600\,nm}$	DMADP (pmol/mg cells)	
		− IPTG	+ IPTG
TG1 wild-type	1.38 ± 0.13	0.21 ± 0.05	0.23 ± 0.04
TG1 (pUC18)	1.02 ± 0.14	0.40 ± 0.19	0.27 ± 0.05
TG1 (pQE50)	1.35 ± 0.11	0.17 ± 0.03	0.14 ± 0.03
TG1 (pUC227ORF1)	0.90 ± 0.06	2.24 ± 1.00	2.79 ± 0.77
TG1 (pUC227ORF1-2)	0.30 ± 0.16	0.31 ± 0.04	0.57 ± 0.07
TG1 (pQE227ORF2)	1.35 ± 0.25	0.23 ± 0.05	0.14 ± 0.07
TG1 (pQE227ORF3)	1.40 ± 0.16	0.12 ± 0.03	0.10 ± 0.02

The DMADP content was determined by gas chromatography after acid hydrolysis into isoprene in 50 mg sediments of overnight liquid cultures grown in minimal glucose containing M9 medium supplemented with 1 mM pyruvate. In order to monitor growth, the $OD_{600\,nm}$ of the overnight culture was determined from a separate aliquot before centrifugation.

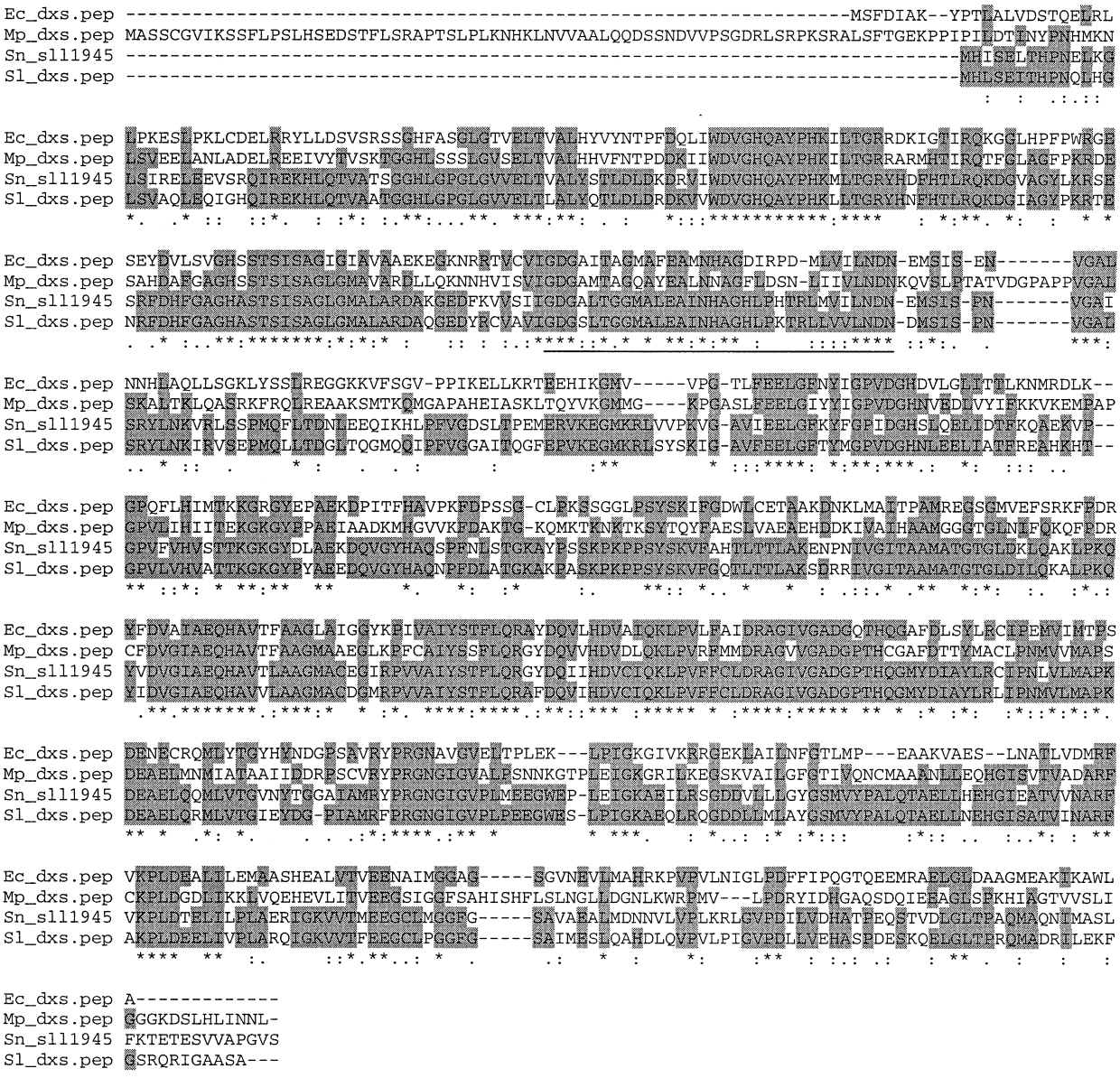

Fig. 3. Comparison of the deduced amino acid sequences of *dxs* genes. Sequences shown in the alignment are from *E. coli* (Ec_dxs.pep, P77488), *M. piperita* (Mp_dxs.pep, G3114573), *Synechocystis* sp. PCC 6803 (Sn_sll1945, P73067) and from *S. leopoliensis* (Sl_dxs.pep, this work). The calculated (ClustalW version 1.74) identities (*), conservative exchanges (:) and similarities (.) in all four sequences are shown below the alignment. The putative thiamine diphosphate binding site is underlined. Amino acid residues identical to the *S. leopoliensis* sequence are marked in gray.

after the end of the *dxs* gene by a PCR reaction using the oligonucleotides 2for, 2rev, 3for, 3rev, 4for and 4rev (see Section 2). This allowed for the cloning of the different ORF behind the *lacZ* promoter of pUC18 or pQE50, which was proven by DNA sequencing (pUC227ORF1, pUC227ORF1-2, pQE227ORF2, pQE227ORF3, see Fig. 2).

3.9. Monitoring the DMADP content of E. coli strains harboring expression fusions of the identified dxs operon

IDP and the isomeric DMADP are the central intermediates of the isoprenoid biosynthesis from which all other isoprenoids in the cell derive. With the aim to prove the involvement of the identified cyanobacterial *dxs* operon in isoprenoid biosynthesis, the DMADP content of transconjugant *E. coli* strains harboring either pUC18, pQE50 without insert or the plasmids pUC227ORF1, pUC227ORF1-2, pQE227ORF2 or pQE227ORF3 with partial inserts of the *dxs* operon fused to the IPTG dependent *lacZ* promoter of the vectors was analyzed. The level of DMADP in wild-type *E. coli* TG1 as well as in the transconjugant with the insertless pUC18 or pQE50 was comparably low at 0.14–0.40 pmol/mg cells (Table 1). Introduction of pUC227ORF1 carrying exclusively cyanobacterial *dxs* led to an approximately 5–8 times increase in the cellular DMADP content, indicating the functionality of ORF1 as *dxs* gene. However, this stimulation was visible already with the groundlevel expression of the gene. Induction by external IPTG resulted only in a slight additional increase in the DMADP content which is probably due to the fact that one of the 4–5 enzymes in the chain from DXP to DMADP is limiting further enhancement of DMADP synthesis. However,

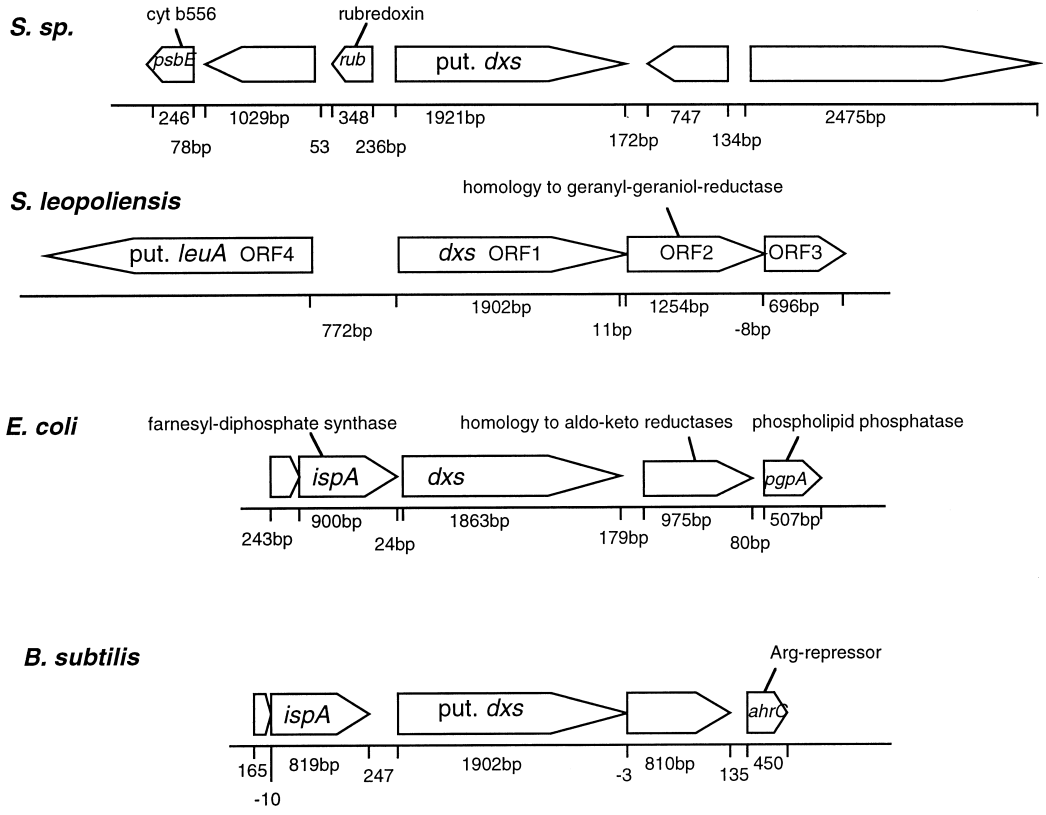

Fig. 4. Organization of *dxs* loci. The *dxs* loci are from *Synechocystis* sp. PCC 6803 (deduced from [24]), *S. leopoliensis* SAUG 1402-1 (this work), *E. coli* [9] and *B. subtilis* (deduced from [29]). The lengths of the genes and of the gaps between the genes are given below and known gene products are indicated above the organization maps.

the fact that the DMADP synthesis could be stimulated by the introduction of *dxs* expression fusion indicates that the Dxs is one of the rate limiting enzymes in the biosynthesis of isoprenoids, at least in bacteria. Moreover, the increased amount of DMADP indicated that the pathway from DXP to DMADP is not feedback-inhibited by one of the products of the pathway. In a quite recently published work, the *dxs* gene of *Synechocystis* sp. 6803 was cloned into a lycopene producing *E. coli* strain in order to monitor the enhancement of isoprenoid biosynthesis [28] via the measurement of the C_{40} isoprenoid lycopene. In contrast to the 5–8 times increased DMADP amount observed in the strains with the cloned *dxs* of the present study, these authors could only observe an increment by a factor of two in the lycopene concentration of their transconjugant strains probably due to the fact that the biosynthesis of lycopene includes a series of additional enzymatic steps after the biosynthesis of DMADP from DXS.

The expression of the construct harboring ORF1 in combination to ORF2 somehow influences growth (Table 1). In all attempts made, this strain grew significantly slower and therefore, the DMADP content is not comparable to that of the other strains. Thus, it is slightly enhanced as compared to the DMADP content of the insertless plasmid containing strains by a factor of two at the highest. Because of the slower growth, ORF2 seems to be expressed in this fusion. However, the expressed polypeptide or the product of the resulting enzyme might cause reduced growth. In addition, introduction of pQE227ORF2 carrying an expressible copy of ORF2 did not influence the DMADP content of the cells compared to the wild-type. The same was true for ORF3. Therefore, a direct involvement of ORF2 and ORF3 in the biosynthesis of DMADP cannot be gathered from the present experiments.

From these expression studies, it can be clearly concluded that the identified ORF1 is indeed encoding the *dxs* gene. This is the first clear-cut proof that this gene is functionally involved in the basic reactions of isoprenoid biosynthesis even in the phototrophic cyanobacteria. This is also a hint that the non-mevalonate pathway found in chloroplasts has probably entered the plant cell ancestor via the green symbiont and the *dxs* was subsequently integrated in the nucleus [10].

Acknowledgements: This work was kindly supported by Grants of the Deutsche Forschungsgemeinschaft to W. Zimmer (No Zi 337/4-3). The authors thank Dr J.-P. Schnitzler for helpful discussions and Prof. Dr H. Rennenberg for critical reading of the manuscript.

References

[1] Rohmer, M., Knani, M., Simonin, P., Sutter, B. and Sahm, H. (1993) Biochem. J. 295, 517–524.
[2] Rohmer, M., Seemann, M., Horbach, S., Bringer-Meyer, S. and Sahm, H. (1996) J. Am. Chem. Soc. 188, 2564–2566.
[3] Schwender, J., Seemann, M., Lichtenthaler, H.J. and Rohmer, M. (1996) Biochem. J. 316, 73–80.
[4] Lichtenthaler, H.K., Rohmer, M. and Schwender, J. (1997) Physiol. Plant. 101, 543–652.
[5] Bouvier, F., d'Harlingue, A., Suire, C., Backhaus, R.A. and Camara, B. (1998) Plant Physiol. 117, 1423–1431.
[6] Lichtenthaler, H.K. (1999) Annu. Rev. Plant Physiol. Plant Mol. Biol. 50, 47–65.

[7] Kreuzwieser, J., Schnitzler, J.-P. and Steinbrecher, R. (1999) Plant Biol. 1, 149–159.
[8] Mandel, M.A., Feldmann, K.A., Herrera-Estrella, L., Rocha-Sosa, M. and Leon, P. (1996) Plant J. 9, 649–658.
[9] Lois, L.M., Campos, N., Putra, S.R., Danielsen, K., Rohmer, M. and Boronat, A. (1998) Proc. Natl. Acad. Sci. USA 95, 2105–2110.
[10] Lange, M., Wilding, M.R., McCaskill, D. and Croteau, R. (1998) Proc. Natl. Acad. Sci. USA 95, 2100–2104.
[11] Therisod, M., Fischer, J.C. and Estramareix, B. (1981) Biochem. Biophys. Res. Commun. 98, 374–379.
[12] Hill, R.E., Himmeldirk, K., Kennedy, I.A., Pauloski, R.M., Sayer, B.G., Wolf, E. and Spenser, I.D. (1996) J. Biol. Chem. 271, 30426–30435.
[13] Takahashi, S., Kuzuyama, T., Watanabe, H. and Seto, H. (1998) Proc. Natl. Acad. Sci. USA 95, 9879–9884.
[14] Kuzuyama, T., Takahashi, S., Watanabe, H. and Seto, H. (1998) Tetrahedron Lett. 39, 4509–4512.
[15] Lange, B.M. and Croteau, R. (1999) Arch. Biochem. Biophys. 365, 170–174.
[16] Schwender, J., Müller, C., Zeidler, J. and Lichtenthaler, H.K. (1999) FEBS Lett. 455, 140–144.
[17] Disch, A., Schwender, J., Müller, C., Lichtenthaler, H.K. and Rohmer, M. (1998) Biochem. J. 333, 381–388.
[18] Wain-Hobson, S., Sonigo, P., Danos, O., Cole, S. and Alison, M. (1985) Cell 40, 9–17.
[19] Knauf, V.C. and Nester, E.U. (1982) Plasmid 8, 45–54.
[20] Sambrook, J., Fritsch, E. and Maniatis, T. (1989) Molecular Cloning, 2nd edn., Vol. 1-3, Cold Spring Harbour Laboratory, Cold Spring Harbour, New York.
[21] Zimmer, W., Brüggemann, N., Emeis, S., Giersch, C., Lehning, A., Steinbrecher, R. and Schnitzler, J.-P. (1999) (submitted).
[22] Lehning, A., Zimmer, I., Steinbrecher, R., Brüggemann, N. and Schnitzler, J.-P. (1999) Plant Cell Environ. 22, 495–504.
[23] Shine, J. and Dalgarno, L. (1975) Nature 254, 34–38.
[24] Taneko, T., Sato, S., Kotani, H., Tanaka, A., Asamizu, E., Nakamura, Y., Miyajima, N., Hirosawa, M., Sugiura, M., Sasamoto, S., Kimura, T., Hosouchi, T., Matsuno, A., Muraki, A., Nakazaki, N., Naruo, K., Okumura, S., Takeuchi, C., Wada, T., Watanabe, A., Yamada, M., Yasuda, M. and Tabata, S. (1996) DNA Res. 3, 109–136.
[25] Hawkins, C.F., Borges, A. and Perham, R.N. (1989) FEBS Lett. 255, 77–82.
[26] Addlesee, H.A., Gibson, L.C., Jensen, P.E. and Hunter, C.N. (1996) FEBS Lett. 389, 126–130.
[27] McCormack, T. and McCormack, K. (1994) Cell 79, 1133–1135.
[28] Harker, M. and Barmley, P.M. (1999) FEBS Lett. 448, 115–119.
[29] Mizumo, M., Masuda, S., Takemaru, K., Hosono, S., Sato, T., Takeuchi, M. and Kobayashi, Y. (1996) Microbiology 142, 3103–3111.