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Molecular Cloning, Expression and Characterization of the First Three Genes in the Mevalonate-Independent Isoprenoid Pathway in *Streptomyces Coelicolor*

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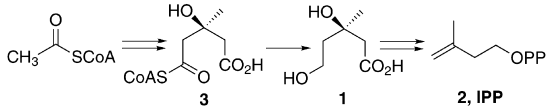
Abstract—The mevalonate-independent biosynthetic pathway to isopentenyl diphosphate and dimethylallyl diphosphate, the universal precursors to the isoprenoids, operates in eubacteria, including *Escherichia coli*, in algae, and in the plastids of higher plants. A search of the Sanger Centre *Streptomyces coelicolor* genome database revealed open reading frames with ca. 40–50% identity at the deduced amino acid level to the first three *E. coli* enzymes of this pathway, corresponding to deoxyxylulose phosphate synthase, deoxyxylulose phosphate reductoisomerase and 2-*C*-methyl erythritol 4-phosphate cytidylyltransferase. The *S. coelicolor* genes have been cloned and expressed in *E. coli*, and the recombinant proteins characterized physically and kinetically. The presence of the corresponding enzyme activities in extracts of *S. coelicolor* CH999 further supports the operation of the mevalonate-independent pathway in this organism.

Introduction

For many years, following the discovery in 1956 that mevalonic acid (**1**) is a precursor of cholesterol, the central dogma of isoprenoid biosynthesis was that all terpenoid natural products, of whatever structure—from monoterpenes to triterpenes—and from whatever source—from microbes to mammals—were derived from the universal precursor, mevalonic acid.[1] All the steps involved in the conversion of mevalonate to the biological isoprene unit, isopentenyl diphosphate (**2**, IPP), have been identified and the relevant enzymes thoroughly characterized (Scheme 1). Given the widespread occurrence of the mevalonate pathway, it was therefore especially startling when in the early 1990s two research groups independently discovered a second biochemical pathway to IPP that involves neither acetate nor mevalonate.[2] The mevalonate-independent pathway was shown to begin with pyruvate and glyceraldehyde-3-phosphate (G-3-P) (Scheme 2). Subsequent investigations have established that this alternative pathway is not a rare exception, but in fact occurs widely in a range of prokaryotic organisms, in algae, and in the chloroplasts or plastids of numerous higher plants.

The first biochemical step in the mevalonate-independent pathway involves the thiamin diphosphate-dependent condensation of pyruvate and D-glyceraldehyde-3-phosphate to give the five-carbon sugar derivative, D-1-deoxyxylulose-5-phosphate (**4**, DXP). (Scheme 2) Interestingly, the free alcohol, 1-deoxyxylulose (**5**, DX), has been known for many years, having previously been isolated from *Streptomyces hygroscopicus*, although the function of this five-carbon ketose had been completely obscure.[3] The structural genes for DXP synthase were first cloned and expressed from *E. coli*[4a] and from peppermint (*Mentha x piperita*).[4b] The *E. coli* DXP synthase shows pronounced similarity to several demonstrated or putative DXP synthases from a variety of bacterial and plant species. Notably, DXP has also recently been shown to be a key intermediate in formation of both pyridoxol phosphate (vitamin B_6)[5] and thiamin (vitamin B_1) itself in *E. coli*.[6]



Scheme 1. Conversion of acetyl-CoA to IPP by way of mevalonate (**1**).

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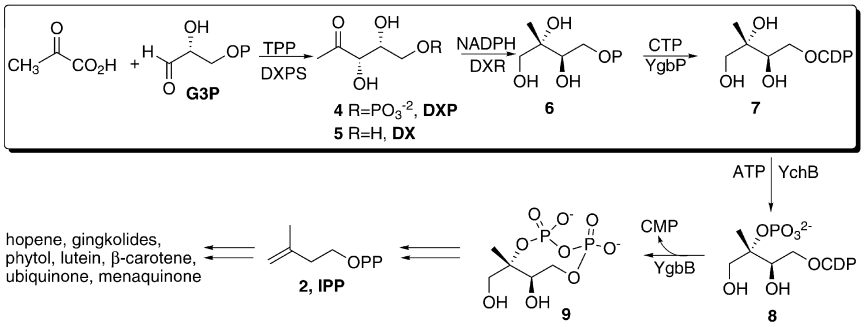


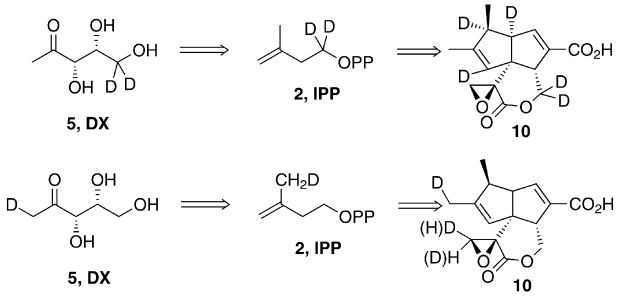
Scheme 2. Mevalonate-independent biosynthetic pathway to IPP and isoprenoids, initiated by the TPP-dependent condensation of pyruvate and G-3-P to give D-1-deoxyxylulose-5-phosphate (**4**, DXP).

In the next step in the mevalonate-independent pathway to IPP, DXP is rearranged to 2-*C*-methyl-D-erythritol-4-phosphate (**6**, MEP) by an NADPH-dependent reductoisomerase (DXP reductoisomerase).[7] MEP has been shown to undergo CTP-dependent conversion to 4-diphosphocytidyl-2-*C*-methylerythritol (**7**, CDP-ME) catalyzed by the *E. coli* protein, MEP cytidylyltransferase.[8] Very recently, it has been established that the 2-hydroxyl group of **7** is phosphorylated by the *ychB* gene product, CDP-ME kinase, to produce 4-(cytidine 5′-diphospho)-2-*C*-methyl-D-erythritol-2-phosphate (**8**, CDP-ME2P).[9] The last identified step in the mevalonate-independent pathway involves the conversion of **8** to 2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate (**9**) with concomitant elimination of cytidine 5′-monophosphate, catalyzed by the *E. coli* YgbB protein.[10] The steps from **9** to isopentenyl diphosphate are as yet unknown and are the object of intensive investigation in several laboratories. Interest in the enzymes of this pathway has been further heightened by the possibility that they might serve as targets for antibacterial or antimalarial drug therapy.[11]

As part of our own long-standing interest in the biosynthesis of isoprenoids, we reported in 1981 the incorporation of [UL-$^{13}C_6$]glucose into a sesquiterpene metabolite of *Streptomyces* UC5319, the antibiotic pentalenolactone (**10**).[12] Although the observed pattern of enhancements and couplings in the ^{13}C NMR spectrum of **10** was mostly as predicted for formation of pentalenolactone by the classical mevalonate pathway, an anomalous set of ^{13}C–^{13}C couplings could not be satisfactorily explained. The eventual discovery of the novel pyruvate/G-3-P route to IPP cast these earlier observations on the labeling of pentalenolactone in an entirely new light. Indeed, all of our earlier reported results could be crisply accounted for by operation of the mevalonate-independent pathway to generate IPP and derived isoprenoid metabolites. In support of this proposal, feeding experiments with *Streptomyces* UC5319 confirmed that labeled 1-deoxyxylulose (**5**) can serve as a specific precursor of **10**.[13] (Scheme 3) In the meantime, Seto has investigated isoprenoid biosynthesis in several *Streptomyces* species, finding evidence for both the mevalonate and the mevalonate-independent routes to IPP and that these two pathways can sometimes co-exist in the same species.[14]

Streptomyces are filamentous, Gram-positive soil bacteria. The Streptomycetes and related actinomycetes are the source of two-thirds of all antibiotics. By far the most thoroughly studied member of this genus is *Streptomyces coelicolor*, which has been extensively characterized genetically and serves as a model of morphological and physiological development in mycelial prokaryotes.[15] Hopwood has recently established a detailed physical and genetic map for *S. coelicolor* A3(2) based on a complete set of ordered, overlapping cosmids that cover the entire 8 Mb linear chromosome.[16] Using these cosmids, the sequencing of the *S. coelicolor* genome has been undertaken by the Sanger Centre, and has been mostly completed as of October 2000 with only a few gaps remaining to be completed. The entire sequence is expected to be completed by 2001. Using the translated *E. coli* sequences as a query for a TBLASTN search of the *S. coelicolor* genome database reveals open reading frames with high levels of similarity to the *E. coli* enzymes corresponding to *dxps*, *dxr*, *ygbP* and *ygbB*. Interestingly, in the case of *dxps*, not one, but two distinct open reading frames can be identified in *S. coelicolor*.

We now report the PCR cloning and the expression in *E. coli* of the *S. coelicolor dxps, dxr* and *ygbP* genes, and the subsequent characterization of the respective proteins. In complementary experiments, the activity of the three native enzymes was also determined in cell-free extracts of *S. coelicolor* CH999.



Scheme 3. Conversion of deuterated deoxyxylulose (**5**) to pentalenolactone (**10**) by *Streptomyces* UC5319.

Results

Construction of plasmids and expression of dxps1 and dxps2 from S. coelicolor—A TBLASTN search of the *S. coelicolor* A3(2) genomic database compiled by the Sanger Centre revealed the presence of two apparent open-reading frames with ca. 50% identity at the deduced amino acid level gene to the *E. coli* DXP synthase protein.[17] (Fig. 1) One of these open reading frames, designated *dxps1*, was contained within *S. coelicolor* cosmid St6A5 (SC6A5;[17] nt 16676–18646), while the second, *dxps2* matched sequences at the overlapping ends of cosmids St7B7 (SC7B7;[17] nt 12,742–13,800-end) and SC1C3 (nt 158–1027. nt 1–157 reported for cosmid SC1C3 overlap nt 13,644–13,800 at the 3′-end of SC7B7). In fact, the complete open reading frame for *dxps2* is actually included in the 3′ end of cosmid St7B7. Both of the apparent *Streptomyces* ORFs corresponded to proteins similar in length to the *E. coli* DXS protein and showed a typical transketolase signature motif. After PCR cloning and expression, both sets of *E. coli* BL21(DE3)/pLysS transformants expressed proteins which migrated on 10% SDS–PAGE with the expected relative masses, M_r 71 kDa (DXPS1, predicted: 69.36 kDa) and 70 kDa (DXPS2, predicted: 68.43 kDa), compared to a reference sample of *E. coli* deoxyxylulose phosphate synthase, M_r 68 kDa (predicted: 67.61 kDa) (data not shown). The majority of each of the recombinant proteins was present in the soluble protein fraction.

Purification and physical characterization of DXP synthases—After optimization of the individual expression conditions, both DXP synthases were purified to homogeneity by similar protocols (Table 1). The experimental level of expression of DXPS1 varied from 1–10% of total (soluble) protein in different runs, while expression levels for DXPS2 appeared to be more reproducible as ca. 20% of total soluble protein. DXPS1 also appeared to be somewhat less stable upon storage and frequently rapidly lost activity. The identity of each of the purified proteins was verified by N-terminal sequencing, which gave results (DXPS1: TILENIRGPR*N*LKALSEA; DXPS2: XXLTRITGPR*N*L*N*RLSL) consistent with the deduced amino acid sequence for each protein after removal of the N-terminal methionine.[18] ES–MS analysis of DXPS1 gave a molecular mass of M_D 69,416 ± 13 (calcd: 69,513 Da) and DXPS2 gave a molecular mass of M_D 68,482 ± 14 (calcd: 68,433 Da), again consistent with the absence of the N-terminal Met. Gel filtration on a calibrated Superose 12 Hr 10/30 column gave apparent molecular weights of M_r 162,000 and 145,500 for DXPS1 and DXPS2, respectively, indicating that both were homodimers. Preparative scale incubation of pyruvate and glyceraldehyde-3-phosphate with DXPS2 in the presence of thiamin diphosphate gave 1-deoxyxylulose-5-phosphate (**4**), the structure of which, was directly confirmed by ^{1}H NMR comparison with a synthetic reference sample.[19]

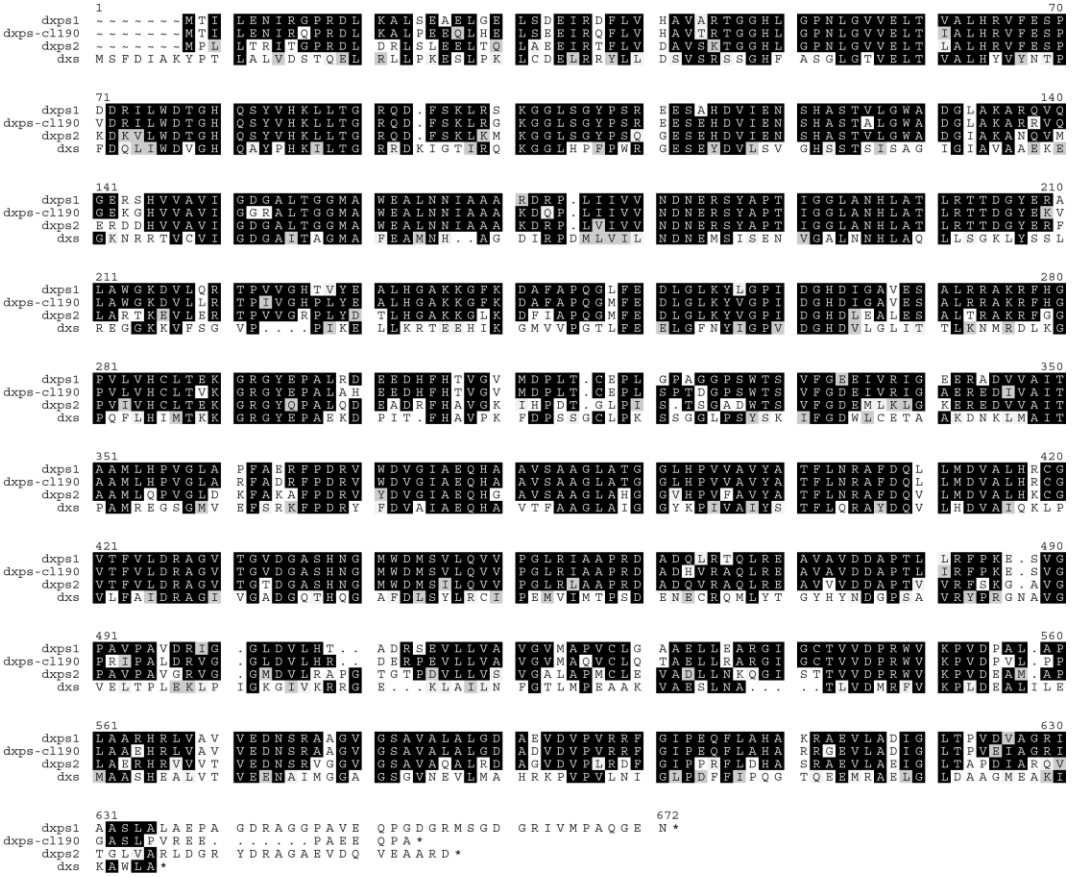


Figure 1. Amino acid sequence alignment of deoxyxylulose-5-phosphate synthases from *S. coelicolor* (dxps1 and dxps2), *Streptomyces* sp. CL190 (cl190), and *E. coli* (dxs). Sequence identities: dxps1/dxps2, 80.1%; dxps1/cl190, 88.7%; dxps1/dxs, 57.7%; dxps2/cl190, 78.9%; dxps2/dxs, 57.3%; cl190/dxs, 55.6%.

Kinetic parameters for DXP synthases

The steady state kinetic parameters for both enzymes were very similar (Table 2). The kinetic parameters of a sample of *E. coli* DXP synthase, which had not been previously published,[20] were similar in magnitude. Both *Streptomyces* enzymes showed only slightly higher turnover numbers than the *E. coli* enzyme and somewhat lower K_m values for each substrate. Both DXP synthases had broad pH optima, with maxima at pH 7.5 and 8.0, respectively.

Construction of plasmids and expression of *S. coelicolor* dxr and ygbP

A TBLASTN search of the *S. coelicolor* A3(2) genomic database revealed the presence of an open-reading frame contained within cosmid St5H4 (SC5H4;[17] nt. 24,696–25,952) with ca. 50.1% similarity and 41.6% identity at the deduced amino acid level to the *E. coli* DXP reductoisomerase[17] and an open-reading frame contained within cosmid StD16A (SCD16A,[17] nt. 4975–5788) that had ca. 44.7% similarity and 36.9% identity to the *E. coli* MEP cytidylyltransferase.[17] After PCR amplification, plasmids were constructed following standard cloning procedures into the pET11 expression vector, followed by transformation into *E. coli* BL21 strains for expression of proteins. While *dxr* was routinely transformed into *E. coli* BL21(DE3)/pLysS competent cells, examination of the *ygbP* sequence with respect to codon usage by *E. coli* revealed several rare codons (proline: CCC; arginine: CGG; glycine: GGG/GGA) typically found in GC-rich genomes. Taking account of this, commercially available *E. coli* BL21(DE3)-RP competent cells were selected as the recipient for the transformation of *ygbP* since this strain was engineered to facilitate the reliable expression of genes restricted by either AGG/AGA or CCC codons.

Purification and physical characterization of DXP reductoisomerase and MEP cytidylyltransferase

The majority of the expressed DXP reductoisomerase was found in the pellet fraction, in the form of inclusion bodies, after cell lysis and centrifugation, with only ca. 10% of expressed reductoisomerase being soluble. After optimization of the expression conditions, the soluble DXP reductoisomerase fraction was purified to ca. 90% homogeneity, as determined by SDS–PAGE, in one chromatographic step using Reactive Red 120 matrix, which contains a ligand resembling the nicotinamide ring. In this way, DXR protein, which is dependent

Table 1. Purification of recombinant *S. coelicolor* DXP synthases (DXPS1 and DXPS2) and MEP cytidylyltransferase (YgbP)

Enzyme	Purification step	Protein (mg)	Activity (nmol DXP or CDP-ME/min)	Spec. Act. (nmol DXP or CDP-ME/min/mg)[a]	Purification (×-fold)
DXPS1	Crude extract	56	62.7	1.12	—
	DE52	12.5	153	12.2	1[b]
	t-Bu-HIC	1.04	63.5	61.1	5[b]
	Resource Q	0.22	27.4	124	10[b]
DXPS2	Crude extract	10.3	3368	327	1
	DE52	2.06	1321	641	2
	t-Bu-HIC	0.5	601	1202	3.7
	Resource Q	0.3	437	1457	4.5
	Gel Filtration	0.14	217	1548	4.7
YgbP	Crude extract	0.018	0.059	0.056	1
	Q-sepharose	0.006	0.12	0.30	5
	Phenyl-sepharose	0.002	0.071	0.77	14
	Superdex HiLoad	0.0007	0.14	3.35	60

[a]Specific activity of purified DXPS1 for different batches of protein ranged from 124 to 1500 nmol DXP/min/mg, with the average ca. 500. The most active batches of protein were used for the kinetic studies (Table 2).
[b]Based on the spec. activity of the DE52-purified DXPS1.

Table 2. Steady state kinetic parameters for recombinant *S. coelicolor* DXP synthases (DXPS1 and DXPS2), DXP reductoisomerase (DXR) and MEP cytidylyl transferase (YgbP) and for the corresponding recombinant *E. coli* DXP enzymes

	S. coelicolor			*E. coli*	
Enzyme	Substrate	K_m (mM)	k_{cat} (s^{-1})	K_m (mM)	k_{cat} (s^{-1})
DXPS1	Pyruvate	0.30±0.01	1.1±0.1	2.40±0.34[a]	0.8±0.2[a]
	G-3-P	0.60±0.07		0.90±0.27[a]	
DXPS2	Pyruvate	0.57±0.03	1.1±0.1		
	G-3-P	0.20±0.03			
DXR	DXP	0.19±0.01	19.20±0.02	0.72±0.06	21.7±0.02
	NADPH	0.19±0.02			
YgbP	MEP	3.26±0.09	1.4±0.1	0.032±0.003[b]	16.8±0.2[b]
	CTP	1.45±0.03	6.0±0.05		

[a]*E. coli* DXS.[20]
[b]Rohdich et al.[8a] have reported K_m values for recombinant *E. coli* YgbP of 3.14 μM (MEP) and 131 μM (CTP), and a k_{cat} 9.9 s^{-1}, determined using a spectrophotometric assay to monitor the production of inorganic pyrophosphate through enzyme-coupling to the reduction of $NADP^+$ at 37 °C.

upon NADPH as a co-factor, was selectively bound to the matrix and eluted only upon successive application of high salt buffer. After optimization of expression conditions, MEP cytidylyltransferase protein was purified in four steps (Table 1). The identity of the purified MEP cytidylyltransferase was verified by N-terminal sequencing, which gave results (SBESRP-SPAETPATTFXE) consistent with the deduced amino acid sequence after removal of the N-terminal methionine. ES–MS analysis of MEP cytidylyltransferase gave a molecular mass of M_D 27,709±13 (calcd: 27,712 Da) again consistent with the absence of the N-terminal Met.

Kinetic parameters for DXP reductoisomerase and MEP cytidylyltransferase

The *Streptomyces* DXP reductoisomerase (DXR) showed very similar turnover numbers to the *E. coli* enzyme but a slightly lower K_m value for DXP. (Table 2) Comparison of the K_m for MEP of the *S. coelicolor* YgbP with that of the corresponding cytidylyltransferase from *E. coli*, however, showed that the *E. coli* enzyme has a K_m for MEP 100-fold lower than that determined for *S. coelicolor* cytidylyltransferase (Table 2). The K_m for MEP (3 μM) reported earlier[8a] for the *E. coli* YgbP is lower still by yet another factor of 10, although the differences between the two sets of values for the *E. coli* enzyme may reflect differences in the enzyme preparation and assay methods used by the two labs. The reported K_m for CTP of the *E. coli* cytidylyltransferase was also smaller, by a tenth, than the value of K_m determined by us for the *S. coelicolor* enzyme. The *Streptomyces* reductoisomerase showed a narrow pH optimum of 8.0, while that of MEP cytidylyltransferase was broader, with a maximum at pH 7.9. The optimal temperature range of the *S. coelicolor* reductoisomerase was 25–30 °C while the optimal assay temperature for the cytidylyltransferase was 30 °C.

Table 3. K_m values for DXP synthase (DXPS), DXP reductoisomerase (DXR) and MEP cytidylyltransferase (YgbP) in cell-free extracts of *S. coelicolor* CH999

Enzyme	Substrate	K_m (mM)
DXPS	Pyruvate	0.34±0.03
	G-3-P	0.55±0.03
DXR	DXP	0.06±0.006
YgbP	MEP	7.80±0.8
	CTP	0.098±0.005

Activity of mevalonate-independent pathway enzymes in *S. coelicolor* CH999

The activities of native DXP synthase, DXP reductoisomerase and MEP cytidylyltransferase were determined for crude, cell-free extracts prepared from submerged, liquid cultures of *S. coelicolor* CH999. The magnitude of the K_m values for each enzyme obtained from radiochemical assays on the crude extracts were comparable to those of the purified, recombinant enzymes, although the value obtained for the K_m of CTP with crude YgbP was somewhat lower than that of the purified protein (Table 3). Since there are presumably two functional DXP synthases present in *S. coelicolor*, it is not possible to assign the observed activity in the crude extracts to a specific DXP synthase. Comparison of the V_{max} values for crude *S. coelicolor* DXPS, DXR, and YgbP with those of the corresponding recombinant enzymes indicated that each of the proteins dedicated to the mevalonate-independent pathway in *S. coelicolor* are each present at ca. 10^{-4}% of total protein in liquid cultures of this organism. (Table 4).

Discussion

Although isoprenoid metabolites have often been presumed to be relatively rare in *Streptomyces*, in fact several examples of such compounds have been reported, including the antibiotic pentalenolactone and related metabolites,[21] the sesquiterpene alcohol epicubenol,[22] and several prenylated aromatic metabolites (e.g., naphterpin, carquinostatins, and novobiocin).[14] Although the genus does not produce steroids, these Gram-positive eubacteria do produce both menaquinones[14,23] and carotenoids.[24] Very recently, Poralla has reported the isolation of pentacyclic hopanoids from the aerial mycelium of sporulating cultures of *S. coelicolor* A3(2).[25] These triterpene metabolites could not be detected, however, in submerged, liquid culture.

Presence of two DXP synthases

Seto has shown that individual *Streptomyces* species may utilize either the mevalonate or mevalonate-independent pathway for the biosynthesis of isoprenoids, with both pathways being found in some organisms.[14] Seto has also recently reported the sequence of a deoxyxylulose phosphate synthase[17] from *Streptomyces* sp. CL190 and characterized the recombinant DXP synthase.[26] Interestingly, *S. coelicolor* DXPS1 has a

Table 4. Comparison of the activities of *S. coelicolor* CH999 crude cell-free extract with *S. coelicolor* DXP synthase (DXPS), DXP reductoisomerase (DXR) and MEP cytidylyltransferase (YgbP) cloned in *E. coli* BL21 strains

CH999 CFE Activity	Substrate	V_{max} (crude)[a] (nmol/min/mg)	V_{max} (pure)[b] (nmol/min/mg)	V_{max} (pure)/V_{max} (crude)
DXPS	Pyruvate	4×10^{-6}	536	3×10^{7}
DXR	DXP	5×10^{-4}	25,800	5×10^{7}
YgbP	MEP	2×10^{-3}	12,750	7×10^{6}

[a]Apparent V_{max} in the crude, cell-free *S. coelicolor* extract.
[b]V_{max} of the purified, recombinant protein.

slightly higher level of sequence identity to the synthase from *Streptomyces* sp. CL190 than it does to *S. coelicolor* DXPS2. (Fig. 1).

The finding that *S. coelicolor* harbors not one, but two, *dxps* genes, is intriguing. Both encode fully functional proteins and exhibit comparable kinetic parameters for each of the two substrates, pyruvate and glyceraldehyde-3-phosphate. The regulation of the individual genes is not yet known, nor is it known whether either (or both) is essential for growth on minimal media. Interestingly, both *dxps* genes are widely separated on the physical map of the *S. coelicolor* genome, and neither is found close to the apparent structural genes encoding the enzymes for the next two biochemical steps in the IPP pathway, the *dxr* gene, corresponding to the reductoisomerase that is located in *Streptomyces* cosmid SC5H4, and the *ygbP* gene for the MEP cytidylyltransferase in *Streptomyces* cosmid SCD16A. Thus, to date, of five *S. coelicolor* genes implicated in the mevalonate-independent IPP pathway (*dxps1, dxps2, dxr, ygbP, ychB, and ygbB*), only *ygbP* and *ygbB*, which are closely linked in all organisms examined to date, are found together and none of the others are even remotely close to one another in the *S. coelicolor* genome. On the other hand, *dxps1* (SC6A5.17) is located in cosmid SC6A5 within 2855 bp of a 6741-bp cluster of five presumptive isoprenoid synthase genes that encode two putative phytoene/squalene synthases (SC6A5.08 and SC6A5.09), a putative phytoene dehydrogenase (SC6A5.11), a putative polyprenyl synthase (SC6A5.12), and a putative squalene-hopene cyclase (SC6A5.13).[17] Notably, a TBLASTN search of the *S. coelicolor* genome database using the *Saccharomyces cerevisiae* HMG-CoA reductase[17] as a query did not turn up any statistically significant matches, suggesting strongly that this key enzyme of the mevalonate pathway is absent in *S. coelicolor*. In contrast, Seto has purified an HMG-CoA reductase and isolated the corresponding structural gene from *Streptomyces* sp. CL190, an organism which he has shown utilizes both the mevalonate and mevalonate-independent pathways to biosynthesize IPP.[27]

Comparison of activities between *S. coelicolor* crude, cell-free extracts and the purified, recombinant proteins

The activities of the downstream enzymes of the mevalonate-independent IPP pathway have not been previously characterized in any *Streptomyces* species. Although the observation of deoxyxylulose phosphate synthase activity in *S. coelicolor* is significant, the first committed step of the mevalonate-independent pathway is catalyzed by the DXP reductoisomerase. The cloning and expression of the genes corresponding to this step and the subsequent YgbP-catalyzed reaction provides strong evidence that *S. coelicolor* carries the necessary complement of functional genes for the novel pathway. To date, there have been few, if any, reports of the detection of enzyme activities associated with the mevalonate-independent pathway in cell-free preparations from the parent organisms, since most studies have focused on molecular genetic analysis and expression of selected recombinant proteins. The fact that all three activities, DXPS, DXR, and YgbP, can also be detected in cell-free extracts of *S. coelicolor* confirms that these enzymes are indeed active in the native organism. Although comparison of the observed *V*max values for the crude *S. coelicolor* extracts with those of the corresponding purified recombinant proteins indicates that each enzyme is normally present as only a small fraction of total protein in liquid cultures of *S. coelicolor*, the observed K_m values are comparable for native and recombinant proteins, except for crude YgbP which showed a somewhat lower apparent K_m (Tables 3 and 4). More significantly, the levels of activity of each enzyme are sufficiently high to encourage the use of *S. coelicolor* as an experimental vehicle to search for the enzymes of the pathway catalyzing steps after that mediated by YgbB and for detection of the corresponding intermediates that lie between the cyclodiphosphate **9** and IPP, consistent with the demonstrated biosynthesis of isoprenoid metabolites in this and other *Streptomyces* species.[32]

Experimental

Materials

Streptomyces coelicolor A3(2) cosmids St6A5 (SC6A5),[17] St7B7 (SC7B7),[17] St5H4 (SC5H4),[17] and StD16A (SCD16A)[17] were gifts from Professor David A. Hopwood and Dr. Helen Kieser of the John Innes Institute, Norwich, UK. *E. coli* JM109/pJKR2 harboring the *E. coli dxs* gene cloned into pUC18 was prepared by Dr. J. K. Robinson in our lab. The *E. coli dxr* and *YgbP* genes were recloned by PCR, using the published sequences,[17] expressed in *E. coli* as pET-11 constructs, and purified as previously described.[7,8] *E. coli* XL1-Blue, *E. coli* BL21(DE3)/pLysS and *E. coli* BL21(DE3)-RP competent cells were purchased from Stratagene (La Jolla, CA). *S. coelicolor* CH999, a derivative of *S. coelicolor* A3(2) in which the entire *act* cluster encoding the genes for the biosynthesis of the highly blue-pigmented antibiotic, actinorhodin, has been deleted, and in which the biosynthesis of a second red-pigmented antibiotic, undecylprodigiosin, has been blocked by a defined mutation,[28] was a gift from Professor Chaitan Khosla of the Department of Chemical Engineering at Stanford University. Culture media were obtained from Difco (Sparks, MD). Silica TLC plates (250 μm) and preswollen DE52 anion exchange resin were from Whatman (Clifton, NJ). SIL-NHR TLC plates were purchased from Macherey-Nagel (Easton, PA). Cellulose $F_{254+366}$TLC plates (100 μm) were from Merck (White House Station, NJ). The prepacked *t*-butyl hydrophobic interaction column (*t*-but-HIC, 5 mL) was obtained from BioRad (Hercules, CA). Sephadex G-25, Resource Q anion exchange, Q-Sepharose, Phenyl-Sepharose, Hi Load 16/60 Superdex 200 prep grade, HR Superose gel filtration columns, and PD-10 G25 gel filtration columns were purchased from Pharmacia Biotech Corp (Piscataway, NJ). Reactive Red 120 matrix and protease inhibitors were purchased from Sigma (St. Louis, MA). Bradford protein assay reagent was from

BioRad. PCR and sequencing primers were synthesized by International DNA Technology (Coralville, IA). Taq and Pfu DNA polymerases, all restriction enzymes, and DNA ligase were purchased from either Stratagene or Promega (Madison, WI). [2-^{14}C]Pyruvate (15.0, 15.8, or 17.5 mCi/mmol) was purchased from New England Nuclear (Boston, MA). All other chemicals were the highest grade available.

Methods

Standard recombinant DNA manipulations were carried out according to published procedures and as previously described.[29,30] All protein purification steps were carried out at 4 °C. Protein concentrations were determined using the Biorad reagent according to the manufacturers directions, using bovine serum albumin as a standard. PCR purification kits were purchased from Qiagen (Valencia, CA). PCR was carried out with a MiniCyler thermocycler from MJ Research (Waltham, MA), equipped with a HotBonnet. Phosphoimaging was performed using Molecular Imaging Screen-BI on a BioRad GS-363 Molecular Imager System and the data processed using Molecular Analyst software. Liquid scintillation was performed on a Beckman Model LS 5801 liquid scintillation counter using Packard Omni-Fluor scintillation cocktail. Spectrophotometric assays were performed using a Hewlett-Packard 8452A Diode Array spectrophotometer. dsDNA sequencing was performed by the HHMI Biopolymer/Keck Foundation Biotechnology Resource Laboratory, at the Yale University School of Medicine, New Haven, CT using the dideoxy dye terminator method and automated fluorescent sequencing. N-terminal protein sequencing, ES–MS, and MALDI–MS protein analysis were carried out by the Keck Laboratory. Analysis of DNA and protein sequences utilized the Compare, Bestfit, Pileup and other programs in the Wisconsin sequence analysis Package, version 10.0 (Unix), Genetics Computer Group (GCG), Madison, WI. *S. coelicolor* cells were disrupted using a French Pressure Cell Press from SLM-Aminco Thermo Spectronic Instruments (Rochester, NY). The *Streptomyces coelicolor* A3(2) Genomic Database is available on the website of the Sanger Centre, Wellcome Trust Genome Campus, Hinxton, Cambridge CB10 1SA: http://www.sanger.ac.uk/Projects/S_coelicolor/.

Cloning and expression of DXP synthases, DXP reductoisomerase and MEP cytidylyl transferase

Primers for the amplification of each gene by PCR were designed based on their respective entire nucleotide sequences with the forward (5′-end) primers (*dxps1*: 5′-tgctagc*cat**ATG***ACGATTCTGGAGAACATCCGGGGACC-3′; *dxps2*: 5′-atatat*cat**ATG***CCGCTGCTGACCCGCAT-3′; *dxr*: 5′-gatc*cat**ATG***AGCCACAGTCCAGCCCCACTCG-3′; *ygbP*: 5′-gcaaat*cat**ATG***TCTGACGAATCGCGT-3′) incorporating a *Nde* I restriction site (italics) and the reverse (3′-end) primers (*dxps1*: 5′-cactgct*ggatcc*TCAGTTCTCTCCTTGCGCTGGC-3′; *dxps2*: 5′-tatata*ggatcc*TCAGTCGCGCGCGGCCTCCA-3′; *dxr*: 5′-gatc*ggatcc*TCATGCACGGGCCTCCGCCGTCT-3′; *ygbP*: tatata*ggatcc*TCAGAACCCATCGTTCAGCCTC-3′) including a *BamH* I site (italics) 3′-of the stop codon, to facilitate in-frame cloning into the pET11a expression vector. The *Streptomyces* GTG start codons found in each gene were replaced with an ***ATG*** codon in each of the forward primers. PCR conditions: denaturation, 5 min (*ygbP*) or 10 min (*dxps1*, *dxps2* and *dxr*) at 95 °C; 30 (*dxr*) or 40 (*dxps1*, *dxps2* and *ygbP*) cycles of amplification (*dxps1* and *dxps2*: denaturation, 95 °C, 1 min; annealing, 65 °C, 30 s; extension, 72 °C, 4 min; *dxr*: denaturation, 95 °C, 30 s; annealing and extension, 75 °C, 4 min; *ygbP*: denaturation, 95 °C, 1 min 30 s; annealing, 65 °C, 30 s; extension, 72 °C, 3 min) and polishing at 72 °C for 2 min (*ygbP*), 5 min (*dxr*) or 10 min (*dxps1* and *dxps2*); 10% DMSO in 1 X Taq polymerase (*dxps1* and *dxps2*) buffer or 10% DMF and 10% gelatin in 1 X Pfu buffer (*dxr*, *ygbP*). The amplified PCR products were digested with *Nde* I and *BamH* I, ligated into the corresponding sites in pET11a, and used to transform *E. coli* XL1-Blue. The resulting plasmids were purified and digested with *Nde* I and *BamH* I to verify the presence of the desired insert and the sequences of the cloned *dxps1*, *dxps2*, *dxr* and *ygbP* genes were confirmed. *E. coli* BL21(DE3)/pLysS was selected as the expression host for transformation with plasmids pET11-pDXPS1, pET11-pDXPS2, and pET11-pDXR while *E. coli* BL21(DE3)-RP was used as the expression host for transformation with plasmid pET11-pYgbP. Expression of protein in Luria-Bertani medium supplemented with 3 μM ampicillin was induced by addition of IPTG (DXPS1: 0.1 mM; DXPS2 and DXP reductoisomerase: 0.5 mM; MEP cytidylyltransferase: 0.4 mM) at mid-log phase (OD 600 0.7) at 22 °C (DXP synthases), 30 °C (DXP reductoisomerase) or 28 °C (MEP cytidylyltransferase). The cultures were incubated for an additional 4 h (DXP synthases and MEP cytidylyltransferase) or 10 h (DXP reductoisomerase) at their respective induction temperatures.

Purification of DXP synthases

A 500 mL culture of IPTG-induced *E. coli* BL21(DE3)/pLysS/pET11-pDXPS1 or /pET11-pDXPS2 was harvested by centrifugation, washed in buffer A (50 mM Tris–HCl, pH 7.9, 2 mM thiamin diphosphate, 5 mM $MgCl_2$, 1 mM dithiothreitol) supplemented with 5 mM 2-mercaptoethanol, resuspended in buffer A, and lysed by sonication. The clear lysate was obtained by centrifugation and passed through a 2.5×20 cm column of DE52 cellulose equilibrated with buffer B (40 mM Tris–HCl, pH. 7.5, 2 mM thiamin diphosphate, 5 mM $MgCl_2$, 1 mM dithiothreitol). After washing with 200 mL of buffer B, enzyme activity was eluted from the column by a 1-L linear gradient of 0–500 mM NaCl in buffer B at a flow rate of 2 mL/min. Enzyme activity pooled from DE52 was mixed with an equal volume of 2 M ammonium sulfate in buffer B to give a final ammonium sulfate concentration of 1 M and the mixture was applied to a *t*-butyl hydrophobic interaction chromatography column (*t*-but HIC, 5 mL) that had been equilibrated with 1 M ammonium sulfate in buffer B. DXPS1 or DXPS2 was eluted by applying 100 mL of a linear gradient of 1.0–0 M ammonium sulfate in buffer B.

Ammonium sulfate in the pooled active fractions was removed by G-25 gel filtration and the desalted filtrate was applied to a 1-mL buffer-equilibrated Resource Q anion exchange column. The desired enzyme activity was eluted by applying 20 mL of a linear gradient of 0–0.5 M NaCl in buffer B. DXPS2 was further purified by gel filtration on a HiLoad 16/60 Superdex 200 column equilibrated with 0.5 M NaCl in buffer B, at a flow rate of 0.2 mL/min. The progress of each purification was monitored by SDS–PAGE. DXP synthase activity was assayed after each purification step by a modification of the previously described procedure.[4a]

Purification of DXP reductoisomerase

Reactive Red 120 was packed into a glass column (2.5×5 cm) and washed with deionized water until no trace of red dye was found in the flow-through. A 500 mL culture of *E. coli* BL21(DE3)/pLysS/pET11-DXR was harvested by centrifugation, washed and resuspended in 50 mL of buffer C (20 mM Tris–HCl, pH 8.0, 5 mM $MgCl_2$) containing 0.1 mM phenylmethanesulfonyl fluoride and 0.1 mM pepstatin A. Cell lysis was performed by sonication, the clear lysate was obtained by centrifugation and applied onto the column that had been equilibrated with 200 mL of buffer C. After washing with 200 mL of buffer C, DXP reductoisomerase activity was eluted with 2 M NaCl in buffer C, pooled and concentrated using an ultrafiltration unit (Millipore YM-30). Purity of the eluted protein was judged by SDS–PAGE. Reductoisomerase activity was assayed qualitatively after purification as previously described.[7b]

Purification of MEP cytidylyltransferase. Q-Sepharose Fast Flow resin was packed in a column (20×3 cm) and equilibrated with buffer A. A 500 mL culture of IPTG-induced *E. coli* BL21(DE3)-RP/pET11-pYgbP was harvested by centrifugation, washed and resusupended in buffer A supplemented with 0.02% sodium azide, 2 mg lysozyme and 0.1 mg DNAse. Cells were disrupted by sonication, the clear lysate was obtained by centrifugation and loaded onto the column, which was washed with a further 200 mL of buffer A. Enzyme activity was eluted from the column by applying 600 mL of a linear gradient of 0–1 M NaCl in buffer A, at a flow rate of 3 mL/min and pooled. In the next purification step, pooled fractions of desired enzyme activity were mixed with an equal volume of 2 M ammonium sulfate in buffer A to give a final ammonium sulfate concentration of 1 M. This mixture was applied to a pre-packed, HiLoad phenyl-sepharose column (20 mL) that had been equilibrated with 100 mL of 1 M ammonium sulfate in buffer A. The column was washed with 100 mL of 1 M ammonium sulfate in buffer A before the MEP cytidylyltransferase protein was eluted by applying 200 mL of a linear gradient of 1–0 M ammonium sulfate in buffer A, at a flow rate of 2 mL/min. MEP cytidylyltransferase was further purified by gel filtration on a HiLoad 16/60 Superdex 200 column equilibrated with buffer A. Phenyl-sepharose purified enzyme was loaded onto the column and eluted after 115 mL of buffer A, at a flow rate of 0.3 mL/min. The desired enzyme activity was pooled and concentrated from 4 to 0.5 mL using an ultrafiltration unit (Millipore YM-10). The purity of protein was judged at each step by SDS-PAGE and Biorad protein assay. After each purification step, MEP cytidylyltransferase activity was assayed following a previously described protocol.[8a]

Determination of native molecular weight

The native molecular weight of DXPS1 and DXPS2 were determined by gel filtration chromatography on an HR Superose 12 gel filtration column equilibrated with 0.5 mL M NaCl in assay buffer A after calibration with standard molecular weight size markers chymotrypsinogen (25 kDa), albumin (67 kDa), aldolase (158 kDa), catalase (237 kDa).

N-Terminal sequencing and molecular mass determination

Purified samples of DXPS1 were electroblotted to a PVDF membrane and submitted for N-terminal sequencing. The purified samples of DXPS2 and MEP cytidylyltransferase were precipitated with trichloroacetic acid, the pellets were redissolved in 50% acetonitrile in water and submitted for N-terminal sequencing and ES–MS.

Determination of steady-state kinetic parameters for DXP synthases

Assays of DXPS1 and DXPS2 were carried out in DXPS assay buffer (40 mM Tris–HCl, 2 mM TPP, 5 mM $MgCl_2$, 1 mM DTT) at 25 °C. With pyruvate as the variable substrate, the concentration of [2-^{14}C]pyruvate (0.64 mCi/mmol) was varied between 0.24 and 9.23 mM with a fixed concentration of glyceraldehyde-3-phosphate of 8.4 mM. With glyceraldehyde-3-phosphate as the variable substrate, the concentration of glyceraldehyde-3-phosphate was varied between 0.19 and 9.18 mM and the concentration of pyruvate (0.32 mCi/mmol) was fixed at 2.53 mM. The amounts of protein and the length of the incubation were adjusted for each enzyme so as to give no more than 10% conversion. Aliquots of 1 μL were analyzed by TLC[4a] followed by phosphoimaging for 24 h. Production of [2-^{14}C]DXP was quantified using a [2-^{14}C]pyruvate standard. The measured intensities were also corrected by subtraction of background at the same TLC R_f, generated by a blank reaction in which assay buffer was used instead of DXP synthase. The data for the formation of DXP as a function of the concentration of each the variable substrate were fit directly to the Michaelis-Menten equation by non-linear least-squares regression using Kaleidagraph software (Synergy Software).

Determination of steady-state kinetic parameters for DXP reductoisomerase

Assays of the reductoisomerase were carried out in DXR assay buffer A (20 mM Tris–HCl, 5 mM $MgCl_2$) at 25 °C. When DXP was the variable substrate its concentration was varied between 0.1 and 10 mM with a fixed concentration of NADPH of 0.2 mM. Determination of the steady-state parameters for NADPH used a starting NADPH concentration of 0.2 mM with DXP

concentration fixed at 5 mM. In a typical assay, NADPH and DXP were added to 1 mL of DXR assay buffer A and the reaction initiated by addition of 10 μL of DXP reductoisomerase solution and the oxidation of NADPH was continuously monitored spectrophotometrically at 340 nm.

Determination of steady-state kinetic parameters for MEP cytidylyltransferase

Assays of MEP cytidylyltransferase were carried out at 30 °C, using [2-^{14}C]MEP (0.31 mCi/mmol). The MEP concentration was varied between 0.5 and 23 mM with the concentration of CTP fixed at 9 mM and the concentration of CTP was varied between 0.5 and 11.9 mM with [2-^{14}C]MEP concentration fixed at 10 mM. In a typical assay, CTP and MEP cytidylyltransferase were added to YgbP assay buffer (100 mM Tris–HCl, pH 8.0, 5 mM $MgCl_2$, 1 mM DTT) and equilibrated to 30 °C for 1 min. Reactions were initiated by addition of [2-^{14}C]MEP. Aliquots of 1 μl were quenched at appropriate time intervals to allow ca. 10% conversion and the reaction was analyzed by thin layer chromatography on SIL-NHR plates[8a] and quantitated by phosphoimaging.

Enzymatic preparation of [2-^{14}C]MEP

To a 50 mM Tris–HCl solution (pH 8.0) containing 20 mM [2-^{14}C]DXP (15.7 mCi/mmol), prepared according to literature procedure,[19] 15 mM NADPH and 1 mM $MnCl_2$, purified DXP reductoisomerase solution was added. The mixture was incubated at 30 °C for 8 h. After removal of enzymes using an ultrafiltration unit (Millipore, YM-10), the reaction mixture was analyzed by TLC on SIL-NHR plates.[8a] The eluted TLC plate was analyzed by phosphoimaging to quantify the concentration of [2-^{14}C]MEP produced, using a calibration curve generated with a [2-^{14}C]pyruvate standard. In a typical preparation, conversion of [2-^{14}C]DXP to [2-^{14}C]MEP was 85%.

Growth of *Streptomyces coelicolor* CH999

One well-sporulated R2YE-agar[31a] culture of *S. coelicolor* CH999 was used to inoculate 100 mL of sterilized, supplemented liquid minimal media (SMM)[31b] that had been modified slightly from the literature preparation by the omission of glycine and antifoam. The inoculated media was placed in an orbital shaker and incubated at 28 °C with reciprocal shaking (325 rpm) for 72 h. Using this saturated 100 mL culture, 2×400 mL of SMM were in turn inoculated and incubated under the same conditions. *S. coelicolor* cells were harvested by centrifugation (6100 *g*, 1 h), washed twice with minimal volume of disruption buffer (100 mM Tris–HCl, pH 8.0, 20 mM NaCl, 1 mM EDTA, 30% v/v glycerol) and resuspended in disruption buffer to ca. 0.5 g wet cells per mL of buffer. As a control, the *dxr* gene was successfully amplified by PCR of *S. coelicolor* genomic DNA obtained from a 50-μL volume of *S. coelicolor* CH999 cell suspension by the alkaline lysis/acetate precipitation method.[31c] *S. coelicolor* CH999 cells were stored at −80 °C until used.

Preparation of *S. coelicolor* CH999 cell free extract

S. coelicolor cells were thawed and protease inhibitors, leupeptin (4 μM) and pepstatin A (3 μM), were added. Cells were disrupted by passage three times through a French pressure cell at 1000 psi. The clear lysate was obtained by centrifugation (19,500 *g*, 35 min) and desalted by G-25 gel filtration using pre-packed PD-10 columns.

Assay of mevalonate-independent pathway activities in *S. coelicolor* CH999

To determine the activity in *S. coelicolor* of mevalonate-independent pathway enzymes, a 4 g wet cell pellet was resuspended in 10 mL of 100 mM Tris–HCl (pH 8.0) and lysed by passage three times through a French pressure cell at 1000 psi. The clear lysate was obtained upon centrifugation (19,000 *g*, 35 min) and desalted by gel filtration with PD-10 columns, in a total volume of 14 mL of 100 mM Tris–HCl (pH 8.0). The total protein concentration of the desalted lysate was 3 mg/mL. The *S. coelicolor* CH999 crude, cell-free extract in 100 mM Tris–HCl, pH 8.0 (14 mL) was incubated at 30 °C with 7 μM [2-^{14}C]DXP (15.7 mCi/mmol), 1 mM $MnCl_2$ and 3 μM NADPH for 18 h, with reciprocal shaking (200 rpm). Aliquots of 14 μL were analyzed at 2 h intervals by thin-layer chromatography on cellulose, eluting with THF/H_2O (9:1)/0.1% v/v TFA, as an alternative TLC system to the 1-propanol/ethyl acetate/water mixture. The eluted TLC plate was analyzed by phosphoimaging to determine the appearance of a new spot which was identified as MEP by co-elution with a [2-^{14}C]MEP standard.

For determination of K_m for DXP synthase, DXP reductoisomerase and MEP cytidylyl transferase in crude, cell-free extracts of *S. coelicolor* CH999, assays were performed at 30 °C with reciprocal shaking at 200 rpm. Typical total protein content in the cell-free extracts, after de-salting, was 5 mg/mL in 100 mM Tris-Cl. Aliquots of 10 μL were quenched with 20 mM EDTA (pH 8.0) and analyzed by thin layer chromatography on either cellulose (DXP synthase and DXP reductoisomerase assays), eluting with THF/H_2O (9:1)/0.1% v/v TFA, or SIL-NHR (MEP cytidylyltransferase) plates eluting with 1-propanol/ethyl acetate/H_2O (6:1:3) (17). The formation of each product was quantitated by phosphoimaging of the TLC plates, using a [2-^{14}C]pyruvate standard. In the assays of DXPS, the concentration of pyruvate (0.22 mCi/mmol) was varied between 0.05 and 1.66 mM while glyceraldehyde-3-phosphate concentration was fixed at 2.5 mM, and the concentration of glyceraldehyde-3-phosphate was varied between 0.15 and 4.60 mM while pyruvate (0.43 mCi/mmol) concentration was fixed at 2.40 mM. The substrates were mixed with 2 mM thiamin diphosphate, 1 mM $MgCl_2$, 2 mM dithiothreitol, and 1 mM NaCl. To initiate the reaction, this mixture was added to 8 mL of CH999 desalted, cell-free extract in 100 mM Tris–HCl (pH 7.5) at 30 °C. For assays of *S. coelicolor* DXR, [2-^{14}C]DXP (0.42 mCi/mmol) concentration was varied between 17 and 267 μM. [2-^{14}C]DXP was mixed with

1 mM NaCl, 1 mM $MnCl_2$ and 7.40 μM NADPH. To initiate the reaction, this mixture was added to 6 mL of desalted, crude cell-free extract of *S. coelicolor* CH999 in 100 mM Tris–HCl (pH 8.0) at 30 °C. To assay native *S. coelicolor* YgbP, the concentration of [2-^{14}C]MEP (1 mCi/mmol) was varied between 3 and 29 μM with the concentration of CTP fixed at 7 mM; similarly, the concentration of CTP was varied between 0.24 mM and 0.94 mM while the concentration of [2-^{14}C]MEP (0.013 mCi/mmol) was fixed at 30 mM. To initiate the reaction, the substrates in 5 mM $MnCl_2$ and 10 mM NaCl were added to 2 mL of desalted, crude cell-free *Streptomyces* extract in 100 mM Tris–HCl (pH 8.0) at 30 °C.

Uncited reference

Ref 33 is not cited in the text

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References and Notes

1. Tavormina, P. A.; Gibbs, M. H.; Huff, J. W. *J. Am. Chem. Soc.* **1956**, *78*, 4498. Spurgeon, S. L.; Porter, J. W. In *Biosynthesis of Isoprenoid Compounds*; Porter, J. W., Spurgeon, S. L., Eds.; Wiley: New York, 1981; pp 1–46..

2. Rohmer, M. In *Comprehensive Natural Products Chemistry. Isoprenoids Including Carotenoids and Steroids*; Cane, D. E., Vol. Ed.; Barton, D., Nakanishi, K., Meth-Cohn, O., Eds.; Elsevier: Oxford, 1999; Vol. 2, pp 45–67. Rohmer, M. *Nat. Prod. Rep.* **1999**, *16*, 565. Schwarz, M.; Arigoni, D. In *Comprehensive Natural Products Chemistry. Isoprenoids Including Carotenoids and Steroids*; Cane, D. E., Vol. Ed.; Barton, D., Nakanishi, K., Meth-Cohn, O., Eds.; Elsevier: Oxford, 1999; Vol. 2, pp 367–400..

3. Slechta, L.; Johnson, L. E. *J. Antibiot.* **2000**, *29*, 685.

4. (a) Sprenger, G. A.; Schörken, U.; Wiegert, T.; Grolle, S.; De Graaf, A. A.; Taylor, S. V.; Begley, T. P.; Bringer-Meyer, S.; Sahm, H. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 12857. Lois, L. M.; Campos, N.; Putra, S. R.; Danielsen, K.; Rohmer, M.; Boronat, A. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 2105. (b) Lange, B. M.; Wildung, M. R.; McCaskill, D.; Croteau, R. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 2100.

5. (a) Cane, D. E.; Du, S.; Robinson, J. K.; Hsiung, Y.-J.; Spenser, I. D. *J. Am. Chem. Soc.* **1999**, *121*, 7722. (b) Laber, B.; Maurer, W.; Scharf, S.; Stepusin, K.; Schmidt, F. S. *FEBS Lett.* **1999**, *449*, 45.

6. Himmeldirk, K.; Kennedy, I. A.; Hill, R. E.; Sayer, B. G.; Spenser, I. D. *J. Chem. Soc. Chem. Commun.* **1996**, 1187.

7. (a) Duvold, T.; Bravo, J. M.; Pale-Grosdemange, C.; Rohmer, M. *Tetrahedron Lett.* **1997**, *38*, 4769. (b) Kuzuyama, T.; Takahashi, S.; Watanabe, H.; Seto, H. *Tetrahedron Lett.* **1998**, *39*, 4509. Takahashi, S.; Kuzuyama, T.; Watanabe, H.; Seto, H. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 9879. (c) Lange, B. M.; Croteau, R. *Arch. Biochem. Biophys.* **1999**, *365*, 170.

8. (a) Rohdich, F.; Wungsintaweekul, J.; Fellermeier, M.; Sagner, S.; Herz, S.; Kis, K.; Eisenreich, W.; Bacher, A.; Zenk, M. H. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 11758. (b) Kuzuyama, T.; Takagi, M.; Kaneda, K.; Watanabe, H.; Dairi, T.; Seto, H. *Tetrahedron Lett.* **2000**, *41*, 703.

9. (a) Luttgen, H.; Rohdich, F.; Herz, S.; Wungsintaweekul, J.; Hecht, S.; Schuhr, C. A.; Fellermeier, M.; Sagner, S.; Zenk, M. H.; Bacher, A.; Eisenreich, W. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 1062. (b) Kuzuyama, T.; Takagi, M.; Kaneda, K.; Watanabe, H.; Dairi, T.; Seto, H. *Tetrahedron Lett.* **2000**, *41*, 2925.

10. (a) Herz, S.; Wungsintaweekul, J.; Schuhr, C. A.; Hecht, S.; Luttgen, H.; Sagner, S.; Fellermeier, M.; Eisenreich, W.; Zenk, M. H.; Bacher, A.; Rohdich, F. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 2486. (b) Takagi, M.; Kuzuyama, T.; Kaneda, K.; Watanabe, H.; Dairi, T.; Seto, H. *Tetrahedron Lett.* **2000**, *41*, 3395.

11. Jomaa, H.; Wiesner, J.; Sanderbrand, S.; Altincicek, B.; Weidemeyer, C.; Hintz, M.; Turbachova, I.; Eberl, M.; Zeidler, J.; Lichtenthaler, H. K.; Soldati, D.; Beck, E. *Science* **1999**, *285*, 1573. Kuzuyama, T.; Shimizu, T.; Takahashi, S.; Seto, H. *Tetrahedron Lett.* **1998**, *39*, 7913.

12. Cane, D. E.; Rossi, T.; Tillman, A. M.; Pachlatko, J. P. *J. Am. Chem. Soc.* **1981**, *103*, 1838.

13. Cane, D. E. In *Comprehensive Natural Products Chemistry. Isoprenoids Including Carotenoids and Steroids*; Cane, D. E., Vol. Ed.; Barton, D., Nakanishi, K., Meth-Cohn, O., Eds.; Elsevier: Oxford, 1999; Vol. 2, pp 155–200. Unpublished results of Arigoni, D.; Eppacher, S.; Schwarz, M. cited on pp 168–169 of ref 13.

14. Seto, H., Watanabe, H.; Furihata, K. *Tetrahedron Lett. 37*, **1996**, 7979. Orihara, N.; Kuzuyama, T.; Takahashi, S.; Furihata, K.; Seto, H. *J. Antibiot. 51*, **1998**, 676. Orihara, N.; Furihata, K.; Seto, H. *J. Antibiot. 50*, **1997**, 979. Shin-ya, K.; Furihata, K.; Hayakawa, Y.; Seto, H. *Tetrahedron Lett. 31*, **1990**, 6025.

15. Hopwood, D. A.; Chater, K. F.; Bibb, M. J. In *Genetics and Biochemistry of Antibiotic Production*; Vining, L. C., Stuttard, C., Eds.; Butterworth-Heinemann: Philadelphia, 1994; pp 65–102.

16. Redenbach, M.; Kieser, H. M.; Denapaite, D.; Eichner, A.; Cullum, J.; Kinashi, H.; Hopwood, D. A. *Mol. Microbiol.* **1996**, *21*, 77.

17. The following nucleotide sequences can be found in the GenBank database: *E. coli* DXP synthase (*dxs*) (GenBank = -GenBank Accession Number AF035440); *E. coli* DXP reductoisomerase protein (*dxr*) (GenBank = GenBank Accession Number AB013300) *E. coli* MEP cytidylyltransferase protein (*ygbP*) (GenBank = GenBank Accession Number AE000358) *S. coelicolor* cosmid SC6A5 (GenBank = GenBank Accession Number AL049485); *S. coelicolor* cosmid SC7B7 (GenBank = GenBank Accession Number AL009199); *S. coelicolor* cosmid SC1C3 (GenBank = GenBank Accession Number AL023702); *S. coelicolor* cosmid SC5H4 (GenBank = GenBank Accession Number AL355913); *S. coelicolor* cosmid SCD16A (GenBank = GenBank Accession Number AL 078618); *Streptomyces* sp. CL190 DXS (GenBank = Genbank Accession Number AB026631) *Saccharomyces cerevisiae* HMG-CoA reductase (GenBank = Genbank Accession Number AAB667527).

18. The three amino acids designated as asparagines (*italic N*) in the N-terminal sequences are actually aspartates, based on the deduced amino acid sequences of DXPS1 and DXPS2.

19. Taylor, S. V.; Vu, L. D.; Begley, T. P.; Schörken, U.; Grolle, S.; Sprenger, G.; Bringer-Meyer, S.; Sahm, H. *J. Org. Chem.* **1998**, *63*, 2375.
20. Boronat and coworkers have measured a K_m for pyruvate of 2.9 ± 0.5 mM for *E. coli* dXP synthase in 40 mM Tris, pH 7.5 at 37 °C (Querol, J.; Besumbes, O.; Lois, L. M.; Boronat, A.; Imperial, S. 4th European Symposium on Plant Isoprenoids, Barcelona, Spain, April 21–23, 1999) See also Seto et al. ref 27.
21. Martin, D. G.; Slomp, G.; Mizsak, S.; Duchamp, D. J.; Chidester, C. G. *Tetrahedron Lett.* **1970**, 4901. Seto, H.; Sasaki, T.; Uzawa, J.; Takeuchi, S.; Yonehara, H. *Tetrahedron Lett.* **1978**, 4411. Cane, D. E.; Oliver, J. S.; Harrison, P. H. M.; Abell, C.; Hubbard, B. R.; Kane, C. T.; Lattman, R. *J. Am. Chem. Soc.* **1990**, *112*, 4513.
22. Gerber, N. N. *Phytochemistry* **1971**, *10*, 185.
23. Yamada, Y.; Hou, C. F.; Sasaki, J.; Tahara, Y.; Yoshioka, H. *J. Gen. Appl. Microbiol.* **1982**, *28*, 519.
24. Arcamone, F.; Camerion, B.; Franceschi, G.; Penco, S. *Gazz. Chim. Ital.* **1970**, *100*, 581. Kato, F.; Hino, T.; Nakaji, A.; Tanaka, M.; Koyama, Y. *Mol. Gen. Genet.* **1995**, *247*, 387.
25. Poralla, K.; Muth, G.; Härtner, T. *FEMS Microbiol. Lett.* **2000**, *189*, 93.
26. Kuzuyama, T.; Takagi, M.; Takahashi, S.; Seto, H. *J. Bacteriol.* **2000**, *182*, 891.
27. Takahashi, S.; Kuzuyama, T.; Seto, H. *J. Bacteriol.* **1999**, *181*, 1256.
28. McDaniel, R.; Ebert-Khosla, S.; Hopwood, D. A.; Khosla, C. *Science* **1993**, *262*, 1546.
29. Sambrook, J.; Fritsch, E. F.; Maniatis, T. *Molecular Cloning, A Laboratory Manual*; 2nd ed; Cold Spring Harbor Laboratory: Cold Spring Harbor, 1989.
30. Cane, D. E.; Kang, I. *Arch. Biochem. Biophys.* **2000**, *376*, 354.
31. Kieser, T.; Bibb, M. J.; Buttner, M. J.; Chater, K. F.; Hopwood, D. A. *Practical Streptomyces Genetics*; The John Innes Foundation: Norwich, 2000: (a) p 408; (b) p 413; (c) p 184.
32. After submission of this manuscript, Poulter reported the existence of two open reading frames encoding distinct deoxyxylulose phosphate synthases in *Rhodobacter capsulatus* and described the expression and characterization of the two enzymes: Hahn, F. M.; Eubanks, L. M.; Testa, C. A.; Blagg, B. S. J.; Baker, J. A.; Poulter, C. D. *J. Bacteriol.* **2001**, *183*, 1.