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Monoterpene biosynthesis pathway construction in *Escherichia coli*

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Dedicated to the memory of Professor Jeffrey B. Harborne

Abstract

Four genes encoding sequential steps for the biosynthesis of the spearmint monoterpene ketone (−)-carvone from the C_5 isoprenoid presursors isopentenyl diphosphate and dimethylallyl diphosphate were installed in *Escherichia coli*. Inducible overexpression of these genes in the bacterial host allowed production of nearly 5 mg/l of the pathway intermediate (−)-limonene, which was mostly excreted to the medium such that products of the downstream steps, (−)-carveol and (−)-carvone, were not detected. Assay of pathway enzymes and intermediates indicated that flux through the initial steps catalyzed by geranyl diphosphate synthase and limonene synthase was severely limited by the availability of C_5 isoprenoid precursors in the host. Feeding studies with (−)-limonene, to overcome the flux deficiency, demonstrated the functional capability of limonene-6-hydroxylase and carveol dehydrogenase to produce the end-product carvone; however, uptake and trafficking restrictions greatly compromised the efficiency of these conversions.

Keywords: *Escherichia coli*; Gene overexpression; Spearmint monoterpenes; Carvone biosynthesis; Geranyl diphosphate synthase; Limonene synthase; Limonene-6-hydroxylase; Carveol dehydrogenase

1. Introduction

The relatively small amounts of commercially useful terpenoids and other natural products accumulated by plants have prompted considerable interest in the development of microbial fermentative processes as an alternative approach for the industrial-scale production of these metabolites (Chotani et al., 2000; Schmidt-Dannert et al., 2000; Barkovich and Liao, 2001; Strohl, 2001). As a foundation for such development, it would be useful to determine the baseline capability of an established microbial host (*Escherichia coli*) to produce such a foreign metabolite by installing and testing a short pathway for plant terpenoid biosynthesis for which the enzymology is defined and the requisite genes are available.

For this purpose, the biosynthesis of the simple monoterpene (−)-carvone was chosen as a model. (−)-Carvone is the principal and characteristic component of spearmint essential oil that is synthesized specifically in the epidermal oil glands of this species (*Mentha spicata*) (Gershenzon et al., 1989). In addition, carvone has been shown to exhibit antimicrobial (Pattnaik et al., 1997) and cancer chemopreventative activity (Zheng et al., 1992; Hohl, 1996), and it finds use in insect pest control (Lee et al., 1997) and as an intermediate in the synthesis of antiviral agents (Wang et al., 2001). The pathway to (−)-carvone requires four enzymatic steps from primary metabolism, including geranyl diphosphate synthase, limonene synthase, cytochrome P450 limonene hydroxylase and carveol dehydrogenase, and it encompasses all of the most common reaction types of terpenoid biosynthesis (i.e. prenyltransfer, cyclization,

Abbreviations: CdH, carveol dehydrogenase; DMAPP, dimethylallyl diphosphate; DXP, deoxyxylulose phosphate; FPP, farnesyl diphosphate; FPPS, farnesyl diphosphate synthase; IPP, isopentenyl diphosphate; IPPI, isopentenyl diphosphate isomerase; GPP, geranyl diphosphate; GPPS, geranyl diphosphate synthase; LS, limonene synthase; L6H, cytochrome P450 limonene-6-hydroxylase; MVA, mevalonic acid; Red, cytochrome P450 reductase; WT, wild type

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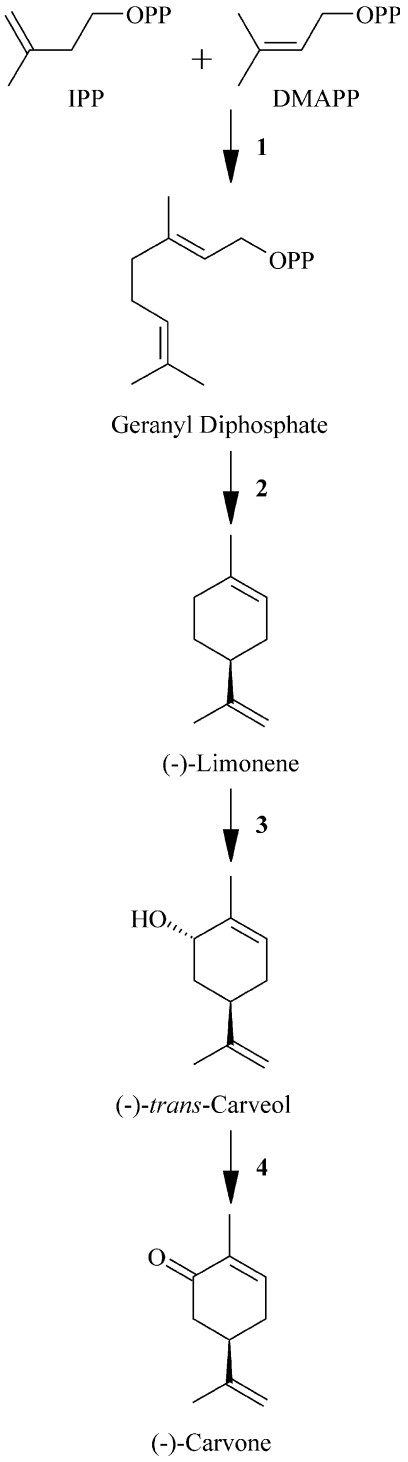

Fig. 1. Pathway for the biosynthesis of (−)-carvone from the primary precursors isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). The indicated enzymes are: 1. geranyl diphosphate synthase; 2. limonene synthase; 3. cytochrome P450 limonene-6-hydroxylase; and 4. carveol dehydrogenase.

oxygenation and redox transformation) (Fig. 1). The enzymology of (−)-carvone biosynthesis has been thoroughly described (Croteau, 1991; Croteau et al., 1991; Wise and Croteau, 1999), and all of the required cDNAs have been acquired and each functionally expressed in *E. coli* by isolation and in vitro assay of the corresponding recombinant enzyme (Colby et al., 1993; Lupien et al., 1999; Haudenschild et al., 2000; Lange et al., 2000; Burke and Croteau, 2002a). Modest success in sesquiterpene and diterpene production by single-step, terpene synthase-catalyzed transformation of endogenous precursors has been achieved in *E. coli* (Hohn and Plattner, 1989; Huang et al., 2001). In this paper, we describe a systematic attempt to install a functional multistep, plant monoterpene biosynthetic pathway in *E. coli*, and we discuss the limitations of this approach when using microbial hosts that are not otherwise enhanced for isoprenoid production.

2. Results and discussion

To determine the inherent capacity of the bacterial host to conduct monoterpene biosynthesis, in the absence of enhanced DXP or MVA pathway flux to C_5 isoprenoid precursors (IPP and DMAPP), the genes for carvone biosynthesis were installed stepwise to evaluate baseline production rates and/or in vivo monoterpene transformation capability. To test the expression of the plant genes in *E. coli*, a range of low and high copy number vectors were evaluated in several host strains, including BLR(DE3) cells carrying the Codon Plus-RIL plasmid. These latter cells transcribe tRNAs for the use of rare codons in *E. coli* that are commonly employed in plant genes (Kleber-Janke and Becker, 2000). High glucose TB media was initially employed, but media enriched in betaine and sorbitol was ultimately utilized to enhance the functional expression of geranyl diphosphate synthase (Burke and Croteau, 2002a) without adverse effect on the expression of other pathway genes. Induction timing and inducer concentration (e.g. 0.1–1.0 mM IPTG) were evaluated, and cultures were monitored over a 4–36 h time course. To assess product formation, the entire bacterial culture was first steam distilled, and the distillate then analyzed by GC–MS. This protocol provides a simple means for the qualitative and quantitative determination of these volatile monoterpenes without interference from *E. coli* metabolites. For the semi-quantitative determination of the non-volatile intermediate GPP, the solvolytic thermally induced (steam distillation) conversion of this metabolite to the volatile, rearranged alcohol linalool (~75% yield) was monitored by GC–MS. The inducible expression of each pathway enzyme was determined in cell-free extracts using established assay methods and by immunoblotting using polyclonal antibodies prepared against the corresponding protein.

As the most logical strategy, we initially installed the pathway in two halves, with the genes for geranyl diphosphate synthase (Burke and Croteau, 2002a) and

limonene synthase (Colby et al., 1993) assembled as one polygenic operon, and limonene hydroxylase (Lupien et al., 1999) and carveol dehydrogenase (Lange et al., 2000) assembled as another. In this way, the production of limonene (and linalool derived from GPP) could be readily determined as a functional measure of the first part of the pathway, and the conversion of exogenous limonene to carveol and carvone could be used as a functional measure of the second part of the pathway. Thus, the two segments of the pathway could be independently optimized before final assembly. To simplify the construction of the first operon, the homodimeric geranyl diphosphate synthase from grand fir (Burke and Croteau, 2002a) was employed rather than the heterodimeric geranyl diphosphate synthase from mint that was initially tested (Burke et al., 1999; Burke and Croteau, 2002b); the kinetics of these two prenyltransferases are very similar (Burke and Croteau, 2002a). For the expression of geranyl diphosphate synthase and limonene synthase, 5′-codons specifying the plastidial targeting peptides of the respective cDNAs were deleted so as to produce mature forms of the two enzymes (Williams et al., 1998; Burke and Croteau, 2002a). It has been shown that geranyl diphosphate synthase does not physically associate with the downstream monoterpene synthases (C.C. Burke and R. Croteau, unpublished); thus, no problem was anticipated in the kinetic coupling of these enzymes from different species. For the second operon, the cytochrome P450 (−)-limonene-6-hydroxylase from spearmint was fused to the ancillary NADPH:cytochrome P450 reductase from peppermint to overcome the deficiency in electron transfer to the eukaryotic cytochrome by the machinery of the procaryotic host (Haudenschild et al., 2000; Wüst et al., 2001).

After construction and sequence verification in pBluescript, the confirmed constructs were transferred into the low copy number expression vector pCL1921 (Lerner and Inouye, 1990), based on the assumption that this vector would provide sufficient, sustained production of the monoterpenes without undue metabolic burden on the host (Jones et al., 2000). The limonene synthase gene alone was also inserted into pCL1921 and transformed into the bacterial host to gain a preliminary assessment of endogenous geranyl diphosphate production. Following induction and growth, the culture was distilled, and GC–MS analysis of the distillate showed barely detectable levels of limonene (production estimated at nM levels in 24 h). As anticipated, *E. coli* did not appear to produce substantial levels of geranyl diphosphate because neither geraniol (derived via host phosphatases) nor linalool (derived by solvolysis of GPP upon distillation) was detected in the culture distillate; this organism lacks a distinct geranyl diphosphate synthase and would be expected to produce only minor levels of the C_{10} prenyl diphosphate en route to higher isoprenalogs by endogenous prenyltransferases (Burke and Croteau, 2002a,b). Next, the vector described above, bearing the grand fir geranyl diphosphate synthase gene inserted upstream of the limonene synthase gene as a polycistronic operon, was transformed into the bacterial host, followed by selection, induction and analysis as before. In this instance, production levels of limonene were only marginally improved and linalool (from GPP) was still not detectable in the distillate, suggesting limitation in the formation of GPP by GPP synthase. Although recombinant limonene synthase was readily observed by immunoblotting and enzyme assay of the corresponding protein extracts, geranyl diphosphate synthase was barely detectable by these means (data not shown), indicating that the low level of expression, coupled to the relatively low k_{cat} of about 2 s^{-1} for this enzyme (Burke and Croteau, 2002a), was restricting synthesis, and that a more efficient vector was required.

The GPPS:LS construct therefore was transferred to pBAD to yield a polycistronic operon as pBAD:GPPS:LS, which was transformed into the host and evaluated as before. In this case, production levels of limonene of about 1 μM in 24 h were achieved but GPP (measured as the derived linalool) was still not detectable, indicating that the supply of GPP in the host was still restricted. To overcome any possible limitation in the expression of geranyl diphosphate synthase under these conditions, the geranyl diphosphate synthase gene was next inserted into the high level expression vector pET37 (i.e. a T7/*lac* promoter-based plasmid with kanamycin resistance) to give pET37:GPPS. The expression of GPPS from pET37 yields activity levels in 10-fold excess of endogenous host FPPS when measured in vitro with IPP and DMAPP as co-substrates (Burke et al., 1999; Burke and Croteau, 2002a). This construct was then cotransformed into *E. coli* BRL(DE3) Codon Plus-RIL cells (chloramphenicol resistance) along with the original limonene synthase construct (pBAD:LS), and the doubly transformed host was selected with a triple antibiotic screen. Induction and analysis as before, led to the overnight production of limonene at levels approaching 40 μM (~5 mg/l) (Fig. 2); however, the GPP precursor was still not detectable (as linalool) in the distillate. These results indicated that GPP did not accumulate (<0.5 μM) under these experimental conditions, and suggested that any of this intermediate that was formed was quantitatively converted to limonene by limonene synthase. Repetition of the experiment, followed by separation of the medium and cells prior to distillation, demonstrated that the bulk of the limonene produced resided in the medium thus indicating that the monoterpene olefin was efficiently exported by the bacterial host.

To evaluate limonene production in greater detail, the expression of both GPPS and LS genes was verified by immunoblot analysis of the corresponding recombinant

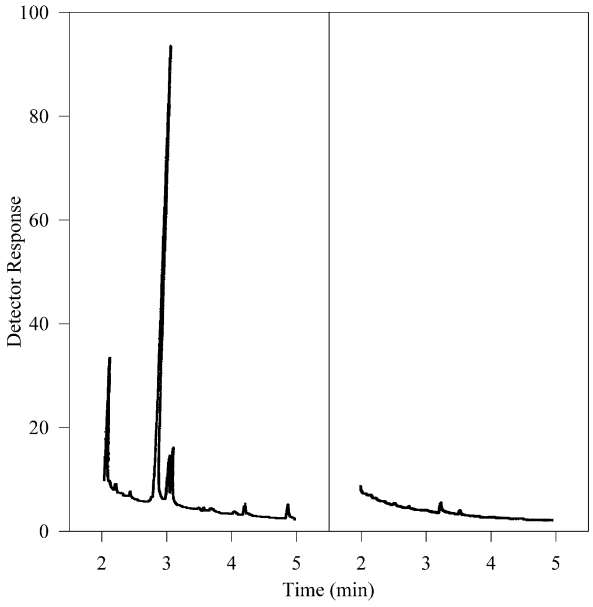

Fig. 2. Capillary GC analysis of the steam distilled products generated by *E. coli* cultures expressing the combination of pET:GPPS and pBAD:LS (left panel), and of control cells expressing both plasmids without inserts (right panel). The peak eluting at R_t 2.86 min is limonene; the small peak eluting after limonene is limonene-1,2-oxide produced by the bacterial host. The production of limonene is conditional on the functional expression of both geranyl diphosphate synthase and limonene synthase. For details of the analysis see Section 4.

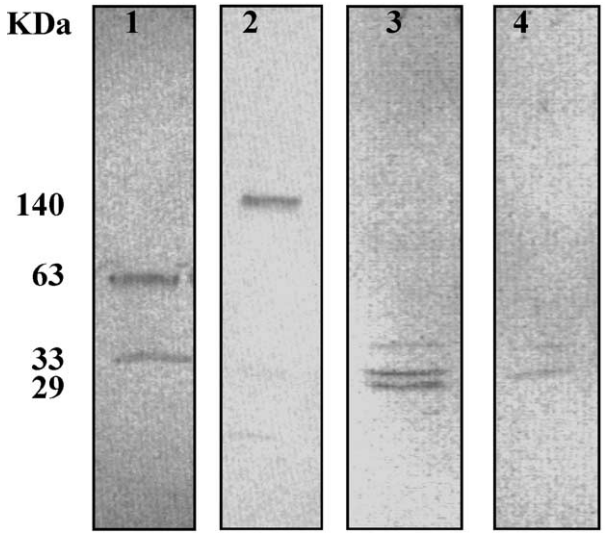

Fig. 3. Immunoblot analysis of protein extracts from *E. coli* cultures expressing the four genes of carvone biosynthesis. Lane 1 illustrates the detection of geranyl diphosphate synthase (33 kDa) and limonene synthase (63 kDa) expressed in the host from the combination of pET:GPPS and pBAD:LS. Lane 2 illustrates the detection of the cytochrome P450 limonene-6-hydroxylase/cytochrome P450 reductase fusion (140 kDa), and Lane 3 illustrates the detection of carveol dehydrogenase (29 kDa), both of which were expressed in the host from pBADS3 containing L6H/Red and CdH. The antigens detected at ~30 and ~35 kDa in Lane 3 are contaminating *E. coli* proteins that were observed in the pBAD empty vector control (Lane 4). Similar empty vector controls for Lanes 1 and 2 showed no extraneous antigens derived from the host. Polyclonal antibodies were prepared in rabbits using the corresponding pure native or recombinant enzyme as antigen. The secondary antibody employed for detection was goat anti-rabbit IgG conjugated to alkaline phosphatase.

proteins in cell free extracts (Fig. 3) and by enzyme assay under optimized conditions (Alonso et al., 1993; Colby et al., 1993; Burke and Croteau, 2002a). Based on calibrated blots and the activity measured, and consistent with prior results on enzyme production levels from these vectors in *E. coli* and established turnover numbers (Williams et al., 1998; Burke and Croteau, 2002a), conservative estimate [measured activity (or enzyme protein$\times k_{cat}$)$\times$incubation time/culture volume] indicated that these doubly transformed cultures were theoretically capable of producing limonene at 1 mM concentrations (at substrate saturation and other optimized reaction conditions). The observations that the host cells produced no detectable intermediate geranyl diphosphate (measured as linalool) and limonene at only 4% of this theoretical maximum now suggested that, although the two enzymatic steps were sufficiently expressed and were apparently efficiently coupled, the production of geranyl diphosphate was rate limiting, presumably by restriction in the supply of C_5 precursors to the GPPS.

Because geranyl diphosphate synthesis requires one DMAPP unit for each condensation with IPP, it was considered possible that DMAPP supply, via the DXP pathway, was deficient in the bacterial host. Therefore, the peppermint isopentenyl diphosphate isomerase (IPPI) gene (often designated *idi*) (Lange et al., 2000) was overexpressed in these producing cells to promote the conversion of IPP to DMAPP (*idi* was transferred to pET37:GPPS to yield pET37:GPPS:IPPI and was then cotransformed with pBAD:LS). A marginal increase in limonene production (<10%), without detectable GPP accumulation, indicated a likely limit in flux to IPP. Although the passive uptake of IPP by *E. coli* is unlikely to be efficient because this C_5 isoprenoid precursor is highly charged, an attempt was made to feed the bacteria with [1-^{14}C]IPP to promote limonene production. This precursor was not appreciably incorporated, and limonene production rates were unchanged.

An attempt was next made to redirect the DXP pathway, which provides precursors for both isoprenoid biosynthesis and the production of thiamine and pyridoxal (Sprenger et al., 1997; Lois et al., 1998; Rohmer, 1999), by direct inhibition of pyridoxal metabolism using hydralazine (Vidrio, 1990), 5-fluorouracil (Aksoy and Baku, 1980) and carboxymethoxylamine (Lopukhov et al., 2002), or by adding thiamine and pyridoxine to the optimized cultures just prior to induction. Results from this set of experiments indicated that hydralazine (at 0.6 μg/ml) was toxic to the cells, thiamine (at 100 μg/ml) slightly enhanced limonene production, and no significant differences were observed with the other compounds.

In all of the above cases in which limonene was produced by the engineered bacterial host, linalool (derived

from geranyl diphosphate) was not detected by GC–MS analysis of the steam distillate. Thus, the small amount of geranyl diphosphate produced in the host was entirely consumed in the formation of limonene or in competing reactions (e.g. by endogenous host FPPS), although the latter possibility (loss by competition) seems very unlikely given the substantial expression levels achieved of GPPS (activity 10-fold in excess of host FPPS) and LS (activity >100-fold in excess of host FPPS). These and all of the above results suggest that the geranyl diphosphate synthase reaction is rate limiting in the process because the co-substrates IPP and DMAPP are not produced in sufficient amounts above the WT host normal cellular supply. Since remedy of this deficiency, by engineering the DXP pathway (Matthews and Wurtzel, 2000; Wang et al., 2000; Kim and Keasling, 2001) or by installing the MVA pathway to increase precursor flux (Campos et al., 2001), was beyond the scope of this work, the remaining studies were conducted under "rate maximized" conditions in which geranyl diphosphate synthase was maintained in the high expression level pET37 vector for independent control of this soluble enzyme. It was thus necessary to transfer the limonene synthase gene to the second polygenic operon in pBAD, which, in its now final form, contained the genes for limonene synthase, cytochrome P450 limonene-6-hydroxylase (as the fusion with cytochrome P450 reductase, i.e. L6H/Red), and carveol dehydrogenase (pBAD:LS:L6H/Red:CdH).

When pET37:GPPS was coexpressed with pBAD containing the operon for the remaining pathway steps, and the cultures induced and analyzed as before, limonene production was observed as expected (at ~40 μM in 24 h), but downstream oxygenated monoterpenes could not be reliably detected. Assessment of expression efficiency of the products of the pBAD pathway operon, by immunoblotting (Fig. 3) and assay of the appropriate soluble or membranous fractions using established methods (Croteau et al., 1991; Colby et al., 1993; Haudenschild et al., 2000), indicated the presence of all of the target enzymes. Based on activity levels, and consistent with prior results on the production of these recombinant enzymes in *E. coli* (Williams et al., 1998; Haudenschild et al., 2000; Lange et al., 2000), conservative estimate (enzyme activity×incubation time/culture volume) indicated that these engineered cells were theoretically capable of producing 10 mM levels of carvone (at saturating levels of limonene and other optimized reaction conditions for each step). The failure to observe production of carveol and/or carvone at operational levels of limonene of approximately 40 μM suggests that the pathway is not kinetically viable (saturation of limonene hydroxylase requires ~150 μM limonene and 350 μM NADPH) and that the very hydrophobic limonene may not be available to the downstream enzymes, either because of the rapid excretion of this product or because the bacterial host lacks the cellular machinery necessary for efficient coupling of the various cytosolic and membranous enzymatic steps. The oil gland secretory cells of mint, in which the carvone pathway normally operates, express abundant binding proteins and lipid transfer proteins dedicated to the task of monoterpene trafficking and secretion (Lange et al., 2000).

In an attempt to evaluate the functional expression of limonene hydroxylase and carveol dehydrogenase in situ, and independent of the restricted production of limonene by GPPS and LS, feeding studies with 1 mM limonene using the engineered host (bearing pBAD:LS:L6H/Red:CdH) were conducted. This concentration of limonene (1 mM) is the theoretical maximum that could be produced by the host from the above noted expression of GPPS and LS, and it represents the approximate operational solubility limit for this monoterpene olefin in the medium (Weidenhamer et al., 1993). Under these conditions, an oxygenated derivative of limonene was produced (Fig. 4) and the recovery of unused substrate exceeded 96%. Given the observed recovery of the substrate, the uptake of limonene was evaluated by repeating the experiment and then separating the medium from the cells via centrifugation prior to the steam distillation step. The low relative concentration of limonene detected in the cells

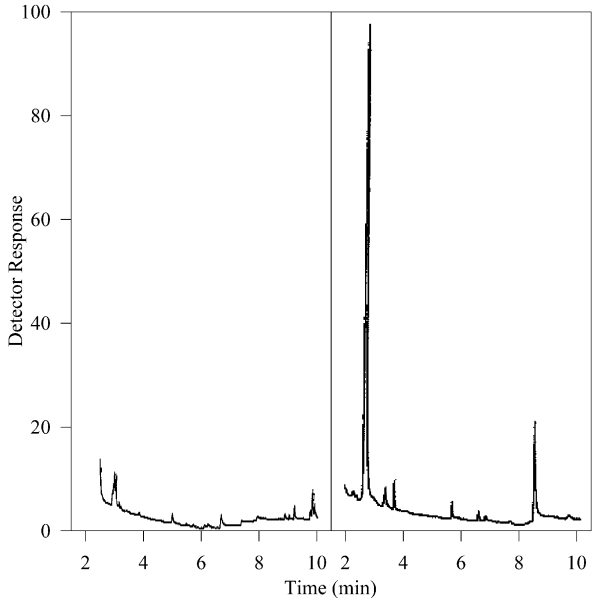

Fig. 4. Capillary GC analysis of the steam distilled products generated by *E. coli* cultures expressing the combination of L6H/Red and CdH from pBAD, in the absence of limonene (left panel) and in the presence of limonene at 1 mM (right panel). The peak eluting at R_t 8.83 min is carvone, and that eluting at R_t 2.86 min is residual limonene; *trans*-carveol, which elutes at R_t 10.28 min, was not detected. The production of carvone is conditional on the functional expression of both limonene-6-hydroxylase and carveol dehydrogenase in the presence of exogenously applied limonene. For details of the analysis see Section 4.

(<200 μM) indicated that this precursor was only poorly taken up or, if taken up, was efficiently excreted consistent with the earlier results.

Analysis of the oxygenated products derived from limonene by *E. coli* cells harboring pBAD:LS:L6H/Red:CdH revealed the presence of a low level of carvone (~2 μM) but the intermediate product carveol could not be detected. Because carveol is observed under conditions of the limonene feed in the presence of L6H/Red alone, the inefficient hydroxylation of limonene to carveol is clearly rate limiting when CdH is added to the operon. The observed operational restriction in the limonene hydroxylation step might be rationalized by the lack of access to this substrate in vivo, in that the activity level of the enzyme measured in vitro was sufficient to have converted all of the limonene (~200 μM) to carveol if this substrate was available.

3. Conclusions

A number of conclusions and cautionary comments can be drawn from the present attempt to transfer a four-step monoterpene biosynthetic pathway of plant origin to a microbial host. First, the endogenous supply of the critical isoprenoid precursors IPP and DMAPP from the DXP pathway of the *E. coli* host is sufficient to support the pathway at only very low flux, and supply appears to be insensitive to the presence of a strong sink (as exemplified by the overexpression of GPPS plus LS, even in the presence of overexpressed IPPI). Recent success in engineering the MVA pathway into *E. coli* has resulted in 20-fold increase in flux to terpenoid products (Martin et al., in press). This general approach to installing a parallel pathway for precursor supply will almost certainly overcome the inherent limitation for terpene production in heterologous hosts. As indicated by the limonene feeding studies, uptake of exogenous monoterpene substrates may limit the efficiency of even single-step biotransformations. Even when multiple, sequential pathway enzymes are satisfactorily expressed, and precursor supply is assured, restrictions may be further imposed by cofactor (e.g. pyridine nucleotide) supply, by rapid export of intermediates, and by the limited ability of the bacterial host to traffic hydrophobic intermediates from the cytosol to membranous hydroxylation sites, and back to the cytosolic sites of subsequent redox metabolism.

4. Experimental

4.1. Substrates and reagents

(−)-Limonene, (−)-carvone, (−)-*trans*-carveol and (±)-linalool were purchased from Aldrich Chemical Co. (Milwaukee, WI), and geranyl diphosphate was synthesized by a literature procedure (Davisson et al., 1985). Thiamine-HCl, pyridoxine-HCl, hydralazine, 5-fluorouracil, and caroboxymethoxylamine were purchased from Sigma Chemical Co. (St. Louis, MO).

4.2. Clones and plasmid constructs

The cDNA clones employed were those originally isolated for geranyl diphosphate synthase from grand fir (Burke and Croteau, 2002a), (−)-limonene synthase from spearmint (Colby et al., 1993), cytochrome P450 (−)-limonene-6-hydroxylase from spearmint (Lupien et al., 1999) and (−)-carveol ((−)-isopiperitenol) dehydrogenase from peppermint (Lange et al., 2000). Truncation studies to delete the plastidial transit peptide of geranyl diphosphate synthase and limonene synthase, and expression of the 'pseudomature' forms in *E. coli*, have been described (Williams et al., 1998; Burke and Croteau, 2002a). Fusion of the limonene-6-hydroxylase with the NADPH:cytochrome P450 reductase from peppermint, and functional expression of the fused species in *E. coli*, have been previously reported (Haudenschild et al., 2000). The NADP-dependent carveol dehydrogenase can be functionally expressed in *E. coli* without modification.

Several individual and multiple gene constructs in different expression plasmids were prepared (Table 1) using standard molecular biological techniques (Sambrook et al., 1989) in order to test the single genes and combinations thereof before final assembly into the two operons required for the production of limonene and the conversion of limonene to carvone. The assembly of these two operons, as described below, illustrates the general strategy involved.

The original pBluescript clones were used as templates for PCR amplification in which unique restriction sites (with generation of new start and stop codons) were appended to each gene (*Kpn*I for the truncated limonene synthase, *Nco*I for the carveol dehydrogenase, and *Xba*I for the cytochrome P450 limonene-6-hydroxylase/NADPH:cytochrome P450 reductase fusion), such that individual modules could be easily reassembled or the entire cassette transferred from one vector to another. The resulting amplicons were transferred individually into the appropriately digested pBAD/*Myc*-HisB Amp^r (Invitrogen, Carlsbad, CA) plasmids to yield pBAD:LS, pBAD:L6H/Red and pBAD:CdH, respectively, which were sequence verified. pBAD:LS was then digested with *Xba*I and the insert transferred to *Xba*I-digested pBAD:L6H/Red to yield pBAD:LS:L6H/Red (designated pBAD:S2), which was sequence verified. This two-gene construct was then digested with *Nco*I and the insert transferred to *Nco*I-digested pBAD:CdH to yield pBAD:LS:L6H/Red:CdH (designated pBAD:S3); this construct was sequence verified as before to confirm

Table 1
Constructs and host systems for expression of geranyl diphosphate synthase (GPPS), isopentenyl diphosphate isomerase (IPPI), limonene synthase (LS), the fusion of limonene-6-hydroxylase and cytochrome P450 reductase (L6H/Red), and carveol dehydrogenase (CdH)

Plasmid	Genes inserted	*E. coli* host strains
pBS[a]	GPPS, LS	JM109, BL21(DE3), BLR(DE3), BLR(DE3) Codon^{+}[f]
pBS	GPPS, LS, CdH	JM109, BL21(DE3), BLR(DE3), BLR(DE3) Codon^{+}
pCWori[b]	L6H/Red	JM109, BL21(DE3), BLR(DE3), BLR(DE3) Codon^{+}
pCL[c]	GPPS, LS	JM109, BL21(DE3), BLR(DE3), BLR(DE3) Codon^{+}
pCL	GPPS, LS, CdH	JM109, BL21(DE3), BLR(DE3), BLR(DE3) Codon^{+}
pBAD[d]	LS	BLR(DE3) Codon^{+}
pBAD	GPPS	BLR(DE3) Codon^{+}
pBAD	L6H/Red	BLR(DE3) Codon^{+}
pBAD	CdH	BLR(DE3) Codon^{+}
pBAD	LS, L6H/Red	BLR(DE3) Codon^{+}
pBAD	LS, L6H/Red, CdH	BLR(DE3) Codon^{+}
pET37[e]	GPPS	BLR(DE3) Codon^{+}
pET37	GPPS, IPPI	BLR(DE3) Codon^{+}

[a] pBluescript-SK +, ColE1ori, lac promoter, Amp^{r}.
[b] pCWori, pBR322ori, lac/tac promoter, Amp^{r}.
[c] pCL1921, pSC101ori, lac promoter, Strep/Spec^{r}.
[d] pBAD/*Myc*-HisB, pBR322ori, T7 promoter, Amp^{r}, arabinose inducible.
[e] pET37b, pBR322ori, T7 promoter, Kan^{r}.
[f] Codon^{+} indicates cells carrying the Codon Plus-RIL Cam^{r} plasmid.

that this operon was capable of directing the synthesis of limonene from geranyl diphosphate and its conversion, via carveol, to carvone.

For the second plasmid, the cDNA clone encoding the truncated version of GPPS (Burke and Croteau, 2002a) was amplified by PCR using primers that introduced a 5′-*Nde*I site (to include the starting methionine codon) and a *Bam*HI site beyond the stop codon and appended $(His)_6$ tag. The purified amplicon was then ligated into *Nde*I/*Bam*HI-digested pET37b Kan^{r} (Novagen, Madison, WI) to yield pET:GPPS. The truncated and $(His)_6$-tagged version of geranyl diphosphate synthase was employed because previous studies have shown this form to be efficiently expressed in *E. coli* (Burke and Croteau, 2002a). The polygenic operons (pBAD:S2 and pBAD:S3) were then individually cotransformed into *E. coli* BLR(DE3) cells carrying the Codon Plus-RIL Cam^{r} plasmid (Stratagene, La Jolla, CA) along with pET:GPPS, and transgenic colonies were selected using a triple antibiotic (Amp–Kan–Cam) screen.

4.3. Overexpression of pathway genes

Small-scale (5 ml) cultures were grown overnight in LB medium at 37 °C under selection conditions and then used to inoculate 250 ml shake flask cultures for monoterpene production. In establishing optimum expression conditions, high glucose TB medium and LB medium supplemented with betaine and sorbitol were tested, induction time and inducer concentration for IPTG (0.1–1.0 mM) and for arabinose (0.002–0.2%) were evaluated, and time courses were run for up to 36 h at 18 or 37 °C. For the final fermentation studies with cotransformed cells, initial growth was conducted at 37 °C in LB medium with 2.5 mM betaine and 0.67 M sorbitol until A_{600} ~0.7 was reached. The temperature was then lowered to 18 °C and the cells were induced with 0.5 mM IPTG (for pET:GPPS) and 0.2% arabinose (for pBAD:S2 or pBAD:S3), and production was monitored for 30 h.

For feeding studies with 1 mM (−)-limonene, this precursor was added (neat) to the cultures at the time of induction, and the incubation was carried out for 18 h at 18 °C. Bacterial packed cell volume was reduced to nearly half by the end of the incubation period, and microscopic examination indicated a significant reduction in cell size compared with identically transformed control cells.

4.4. Assays

To monitor the biosynthesis and/or bioconversion of monoterpenes by the transformed cells, these volatile products were first isolated by direct simultaneous steam distillation-hexane extraction of the cultures using a Likens-Nickerson apparatus (J&W Scientific, Folsum, CA) that was equipped with a standard condenser cooled with ice water (Gershenzon et al., 2000). Geraniol was not detected as a product of geranyl diphosphate, derived by endogenous phosphatase activity of the microbial host; therefore, the non-volatile intermediate geranyl diphosphate was thermally solvolyzed (~75% yield) to the rearranged, volatile alcohol linalool (Bunton et al., 1979) under these distillation conditions (~70% recovery), thus permitting ready analysis. This method for the determination of GPP (detection

limit about 0.5 μM in culture for the present case) has been described previously (Croteau and Cane, 1985). For this purpose, the entire culture was transferred to a 500 ml round bottom flask, and this mixture plus hexane in the solvent-containing side-arm flask were both heated to reflux and distilled for 15 min. The volatile material collected in the hexane fraction was then passed over a short silica gel column which was washed with 1.5 ml hexane to yield limonene in the combined eluate. The column was then washed with a few ml of diethyl ether to provide an eluate containing the oxygenated monoterpenes (linalool derived from GPP, as well as carveol and carvone).

The hexane and ether fractions were diluted or concentrated as necessary prior to analysis of aliquots by capillary GC with FID and by GC-MS using established protocols (Crock et al., 1997; Steele et al., 1998; Schwab et al., 2001). Monoterpene products were identified by comparison of retention times and mass spectra to those of the authentic standards. Quantification of monoterpene products was accomplished by calibration of the system with authentic standards. Overall distillation recoveries ranged from 70 to 95%; this method for monitoring the in situ production or bioconversion of monoterpenes in *E. coli* cultures has been used extensively in the functional screening of terpenoid biosynthetic genes (Lange et al., 2000; McConkey, 2001).

The extraction and assay of all of the target enzymes have been described in detail in the literature for geranyl diphosphate synthase (Burke and Croteau, 2002a), limonene synthase (Williams et al., 1998), limonene-6-hydroxylase/cytochrome P450 reductase fusion (Haudenschild et al., 2000), and carveol dehydrogenase (Croteau et al., 1991; Lange et al., 2000). Protein samples for immunoblotting were prepared by standard protocol (Garfin and Bers, 1989) and were separated under denaturing conditions on 4-15% polyacrylamide gradient gels (Bio-Rad, Hercules, CA). Following electrotransfer to supported nitrocellulose membranes (Bio-Rad), antigens were detected using polyclonal antibodies prepared in rabbits against the corresponding native or recombinant enzyme, along with goat anti-rabbit IgG conjugated to alkaline phosphatase as secondary antibody (Boehringer Mannheim, Indianapolis, IN).

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