The In Vivo Synthesis of Plant Sesquiterpenes by *Escherichia coli*

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Abstract: Three plant genes encoding (+)-δ-cadinene, 5-*epi*-aristolochene, and vetispiradiene cyclases were expressed in *Escherichia coli* to evaluate the potential of this bacterium to synthesize sesquiterpenes in vivo. Various growth temperatures, carbon sources, and host strains were examined to optimize terpene production. The highest levels of sesquiterpene production occurred when the enzymes were expressed in strain DH5α from the trc promoter (Ptrc) of the high-copy plasmidpTrc99A in M9 medium supplemented with 0.2% (v/v) glycerol at 30°C for 5-*epi*-aristolochene and vetispiradiene and 37°C for (+)-δ-cadinene. The highest concentrations of sesquiterpenes observed were 10.3 μg of (+)-δ-cadinene, 0.24 μg of 5-*epi*-aristolochene (measured as (+)-δ-cadinene equivalents), and 6.4 μg of vetispiradiene (measured as (+)-δ-cadinene equivalents) per liter of culture. These sesquiterpene production levels are >500-fold lower than carotenoid production, both of which are synthesized from endogenous *trans*-farnesyl diphosphate (FDP) in *E. coli*. Based on these results, we conclude that the limiting factor for sesquiterpene synthesis in *E. coli* is the poor expression of the cyclase enzyme and not supply of the FDP precursor. © 2001 John Wiley & Sons, Inc. *Biotechnol Bioeng* **75**: 497–503, 2001.

Keywords: isoprenoid; sesquiterpene; cyclase; (+)-δ-cadinene; 5-*epi*-aristolochene; vetispiradiene

INTRODUCTION

Wonderful aromas and potent antimicrobials represent only a few of the attributes of plant terpenes. These compounds have been used for centuries as constituents of spices and traditional herbal remedies in alternative medicine. Along with the advances of modern medicine came a need to find and develop pharmaceuticals to treat diseases. Natural products from plants quickly became a significant source of diversity for the discovery of new small molecules with therapeutic properties, and plant terpenes were among them.

The primary building block for the synthesis of terpenes, isopentenyl diphosphate (IDP), can be synthesized via two pathways: the mevalonate-dependent and the mevalonate-independent pathway (also referred to as the DXP pathway). In prokaryotes, isoprenoids are synthesized primarily via the mevalonate-independent pathway (Boucher and Doolittle, 2000), and in *Escherichia coli* it is used to produce the intermediate *trans*-farnesyl diphosphate (FDP). FDP is the precursor to *trans*-octaprenyl diphosphate (ODP), used in ubiquinone synthesis (Asai et al., 1994) and *cis*, *trans*-undecaprenyl diphosphate (UPD), used in bactoprenol biosynthesis (Apfel et al., 1999).

Sesquiterpenes are formed from the allylic diphosphate substrate FDP by a family of enzymes collectively referred to as sesquiterpene cyclases or synthases. In nature, these enzymes are found in plants, insects, and fungi and are used in the synthesis of phytoalexins (Chen et al., 1995), communication transducers, alarm pheromones (Crock et al., 1997), and insect defense compounds (Steele et al., 1998). Several genes encoding plant sesquiterpene cyclases have been cloned and characterized (Bohlmann et al., 1998). We chose to study sesquiterpene synthesis in a heterologous host, namely *E. coli*, by three plant sesquiterpene cyclases. The plant cyclases were 5-*epi*-aristolochene cyclase (TEAS) from *Nicotiana tabacum* (Facchini and Chappell, 1992), (+)-δ-cadinene cyclase (Cad) from *Gossypium arboreum* (Chen et al., 1995), and vetispiradiene cyclase (HVS) from *Hyoscyamus muticus* (Back and Chappell, 1995).

In studying terpene synthesis in bacteria we hope to facilitate the transition of natural products from discovery to development and commercial production. Terpenes and their derivatives are often difficult to produce in significant yields in higher plants or by synthetic means. Although the research on natural product biosynthesis is still at the gene discovery stage, the explosion of DNA sequence data from genomics research provides an excellent opportunity for the engineering of microorganisms for the synthesis of natural products. Combined with recent advances in enzyme directed

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evolution and combinatorial biocatalysis, this approach may be a valuable alternative to traditional methods of drug discovery, development, and production. In this paper we report on the optimization of sesquiterpenes production in *E. coli* by three plant sesquiterpene cyclases.

MATERIALS AND METHODS

Plasmids and Microbial Strains

Each of the three plant sesquiterpene cyclase genes was cloned into the pBAD24 (Guzman et al., 1995) and pTrc99A (Amann and Abel, 1988) expression vectors. The tobacco TEAS, *Hyoscyamus muticus* HVS, and cotton Cad cyclases were cloned by PCR using pBSK-TEAS (Facchini and Chappell, 1992), cVS1 (Back and Chappell, 1995), and cad1-C1 (Chen et al., 1995) cDNA clones as templates, respectively. The PCR products were cloned into the pCR4-TOPO TA cloning vector (Invitrogen), sequenced, and subcloned using the appropriate restriction endonucleases. The PCR reactions were performed with 30 cycles at 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 2 min and used the *Taq* and *Pow* enzyme mix from the Expand High Fidelity PCR System (Roche Indianapolis). The PCR primers used were as follows (restriction sites underlined and start/stop codons in bold): TEAS, 5′-AGCTCGAATTCC**ATG**GCCTCAGCAGCAGTTGCAAA-3′ and 5′-GCGGTACC**TCA**AATTTTGATGGAGTCCAC-3′; HVS, 5′-AGCTCGAATTCC**ATG**GCCCCAGCTATAGTGATGAG-3′ and 5′-GCTCTAGA**TCA**AATATCAATAGAATCACCACCAA-3′; Cad, 5′-AGCTCGAATTCC**A TG**GCTTCACAAGTTTCTCAAAT-3′ and 5′-GCTCTAGA**TCA**AAGTGCAATTGGTTCAAT-3′. To test the effect of increased flux to FDP on sesquiterpene production, the gene encoding the *E. coli* 1-deoxy-D-xylulose-5-phosphate synthase (*dxs*) was coexpressed with sesquiterpene cyclases. The *dxs* gene, under the control of the arabinose-inducible promoter *araBAD* (P_{BAD}), was cloned into the broad-host-range vector pBBR1MCS-2 (Kovach et al., 1995) using the *Cla* I-*Xba* I DNA fragment from pDdxs (Kim and Keasling, 2001). The genes encoding the tRNAs for the rare *E. coli* arginine (*argU*), isoleucine (*ileY*), and leucine (*leuW*) codons were provided on a pACYC-based plasmid (RIL plasmid, Stratagene). The analysis of sesquiterpene production was carried out in *E. coli* strain DH5α (F^- ϕ80d *lac*ZΔM15 *rec*A1 *end*A1 *gyrA*96 *thi*-1 *hsd*R17($r_k^- > m_k^+$) *sup*E44 *rel*A1 *deo*RΔ(*lac*ZYA-*arg*F)U169) unless otherwise stated.

Media and Culture Conditions

For the cultivation of *E. coli* and optimization of sesquiterpene production, Luria Bertani (LB), 2×YT (16 g tryptone, 10 g yeast extract, 5 g NaCl per liter), and M9 minimal (6 g Na_2HPO_4, 3 g KH_2PO_4, 1 g NH_4Cl, 0.5 g NaCl, 3 mg $CaCl_2$, 1 mL 1 M $MgSO_4$, 0.5% w/v vitamin B1 per liter) media were used. The media were supplemented with the appropriate antibiotics at the following concentrations (ampicillin, 100 μg/mL; chloramphenicol, 50 μg/mL; kanamycin, 50 μg/mL). Inocula for sesquiterpene production experiments were grown in LB broth at 37°C to the midexponential growth phase (0.6–0.7 optical cell density at 600 nm [OD_{600}]). Sesquiterpene production cultures were inoculated (1% v/v) with this seed culture, induced with 13.3 mM arabinose and/or 1 mM IPTG as required and incubated for 24 hr on a rotary shaker (200 rpm), unless otherwise stated. Preliminary experiments carried out in our laboratory showed that coexpression of the RIL plasmid with the Cad cyclase increased sesquiterpene production. Therefore, the RIL plasmid was coexpressed with the pTrc-Cad unless otherwise indicated.

GC-MS Analysis of Sesquiterpenes

For analysis, sesquiterpenes from culture aliquots of 0.75 mL were extracted with an equal volume of ethyl acetate containing the internal standard caryophyllene (Sigma) at a concentration of 0.1 ng/mL. Extractions were carried out in glass vials to avoid contaminants that were extracted from plastic tubes. The organic phase was subjected to gas chromatography electron impact-mass spectrometry (GC-EI-MS) using a Hewlett Packard HP6890 gas chromatograph equipped with a Hewlett Packard 5973 mass selective detector and a HP-5MS capillary column (30M×250μm id × 0.25μm film thickness, Hewlett Packard, Palo Alto). Sesquiterpenes from splitless 1 μL injections were separated using a GC oven temperature program of 80°C for 2 min, a ramp of 30°C/min to 160°C followed by a ramp of 3°C/min to 170°, and finally a ramp of 50°C/min to 300°C. Injector and MS quadrupole detector temperatures were 250°C and 150°C, respectively. To increase the sensitivity and selectivity of detection, the MS was operated in selected ion monitoring (SIM) mode using ions of m/z 105, 161, and 204, which represent the molecular ion and two abundant ions of sesquiterpenes. Based on a Cadinen (Fluka) standard curve, we estimated the detection limit of the instrument in SIM mode to be 0.1 ng/L. Because there are no commercially available analytical standards, the 5-*epi*-aristolochene and vetispiradiene concentrations are reported as (+)-δ-cadinene equivalents based on their relative abundance of the 105, 161, and 204 ions to all ions between 40 and 204. These values were 30.3% (+)-δ-cadinene, 11.2% for vetispiradiene, and 24.3% for 5-*epi*-aristolochene. All concentrations are means of replicates of three independent cultures, and error bars represent ±standard deviations.

RESULTS

Confirmation of the Functional Expression of Terpene Cyclases and the Effect of Expression Vector and Culture Medium on Sesquiterpene Production

To confirm the functional expression of the sesquiterpene cyclase genes, we analyzed the ethyl acetate extracts of cultures of *E. coli* expressing each cyclase from either the IPTG-inducible trc promoter (P_{trc}) of the high copy vector pTrc99A or the arabinose-inducible *ara-BAD* promoter (P_{BAD}) of pBAD24. Sesquiterpene peaks in the GC chromatograms of extracts of cultures expressing the cyclases were initially identified by comparison of chromatograms of cultures extracts not expressing the cyclases. Using the initial culture growth conditions (LB broth and 37°C), sesquiterpenes were not detected in the cultures expressing the cyclases from the pBAD24 expression vector, with the exception of pBADHVS. When the cyclases were expressed from pTrc99A, we observed the production of (+)-δ-cadinene and vetispiradiene (Fig. 1). On optimization of the culture conditions for pH and temperature (see next section), the production of 5-*epi*-aristolochene was also observed. The sesquiterpenes identified by gas chromatography using SIM had retention times of 7.42, 7.62, and 7.78 corresponding to 5-*epi*-aristolochene, vetispiradiene, and (+)-δ-cadinene, respectively. To confirm the identity of the three sesquiterpene peaks, we obtained full scan mass spectra with ethyl acetate extracts that were concentrated by >20-fold under a gentle stream of N_2 gas to increase sensitivity (Fig. 2). The identity of (+)-δ-cadinene was confirmed by comparison to the GC retention time and mass spectrum of the authentic standard. The mass spectrum of 5-*epi*-aristolochene was compared with its published profile and found to be a match (Whitehead et al., 1989). As

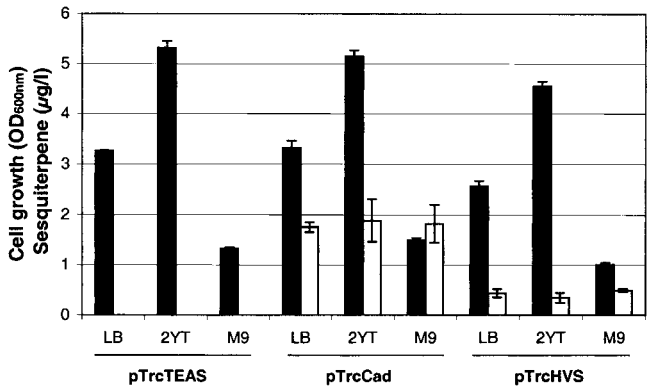

Figure 1. Comparison of the growth and sesquiterpenes production from *E. coli* (DH5α) expressing sesquiterpene cyclases from the pTrc99A vector in LB, 2×YT and M9 + 0.2% glycerol media at 37°C for 24 hr. Growth, black bars; sesquiterpene concentration, white bars. Concentrations of 5-*epi*-aristolochene and vetispiradiene are reported as (+)-δ-cadinene equivalents.

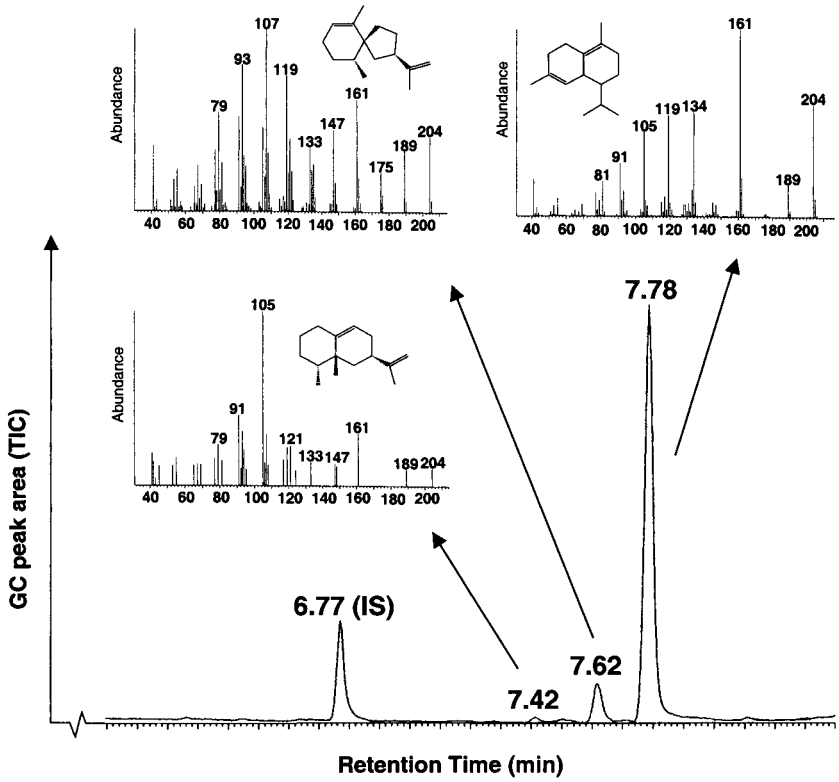

Figure 2. Gas chromatographic analysis and mass spectra of the sesquiterpene cyclase products generated by the pTrcTEAS, pTrcCad, and pTrcHVS clones. IS: caryophyllene internal standard.

previously reported (Back and Chappell, 1996), the clone expressing the HVS produced a single peak with a mass spectrum matching that of sesquiterpenes, which was assumed to be vetispiradiene.

Sesquiterpene production was investigated using nutritionally rich LB and 2×YT media and the minimal phosphate-buffered M9 medium containing 0.2% glycerol. Although the three media gave similar titers, M9 gave the best yields (titer per unit OD) of sesquiterpenes even though the growth of these cultures was two- to five fold lower than of rich media (Fig. 1). In addition, the GC chromatograms of culture extracts from M9 medium showed very few peaks other than the sesquiterpene peaks (Fig. 2) compared with LB or 2×YT cultures, which allowed better detection limits due to low signal noise.

Optimization of Growth Temperature and pH of M9 Medium

To establish the optimal culture conditions for sesquiterpene production in M9 medium, a range of growth temperatures and pHs were examined. The production of 5-*epi*-aristolochene and vetispiradiene increased significantly at lower temperatures, whereas the production of (+)-δ-cadinene was maximal at a higher temperature of 39°C (Fig. 3). In fact, depending on the pH, very little or no 5-*epi*-aristolochene was observed at temperatures above 35°C, and vetispiradiene production also declined significantly at higher temperature. We suspect that, as previously reported for TEAS, the reduced sesquiterpene production at higher temperature was the result of poor cyclase activity due to the formation of insoluble enzymes (Back et al., 1994). The pH of the medium had a considerable effect on 5-*epi*-aristolochene production, where the highest yield was found at pH 7.0 and dropped significantly at a more acidic or basic pH (Fig. 3). The optimal pH for (+)-δ-cadinene and vetispiradiene production was 7.5; however, variation in pH of the vetispiradiene culture had only a minor impact on production.

Effect Carbon Source and Host Strain on Sesquiterpene Production

Using the established growth conditions for M9 medium, various carbon sources and strains of *E. coli* were used to determine their effect on sesquiterpene yields. In all cases, medium containing 0.2% glycerol produced the highest yields of sesquiterpene whereas pyruvate

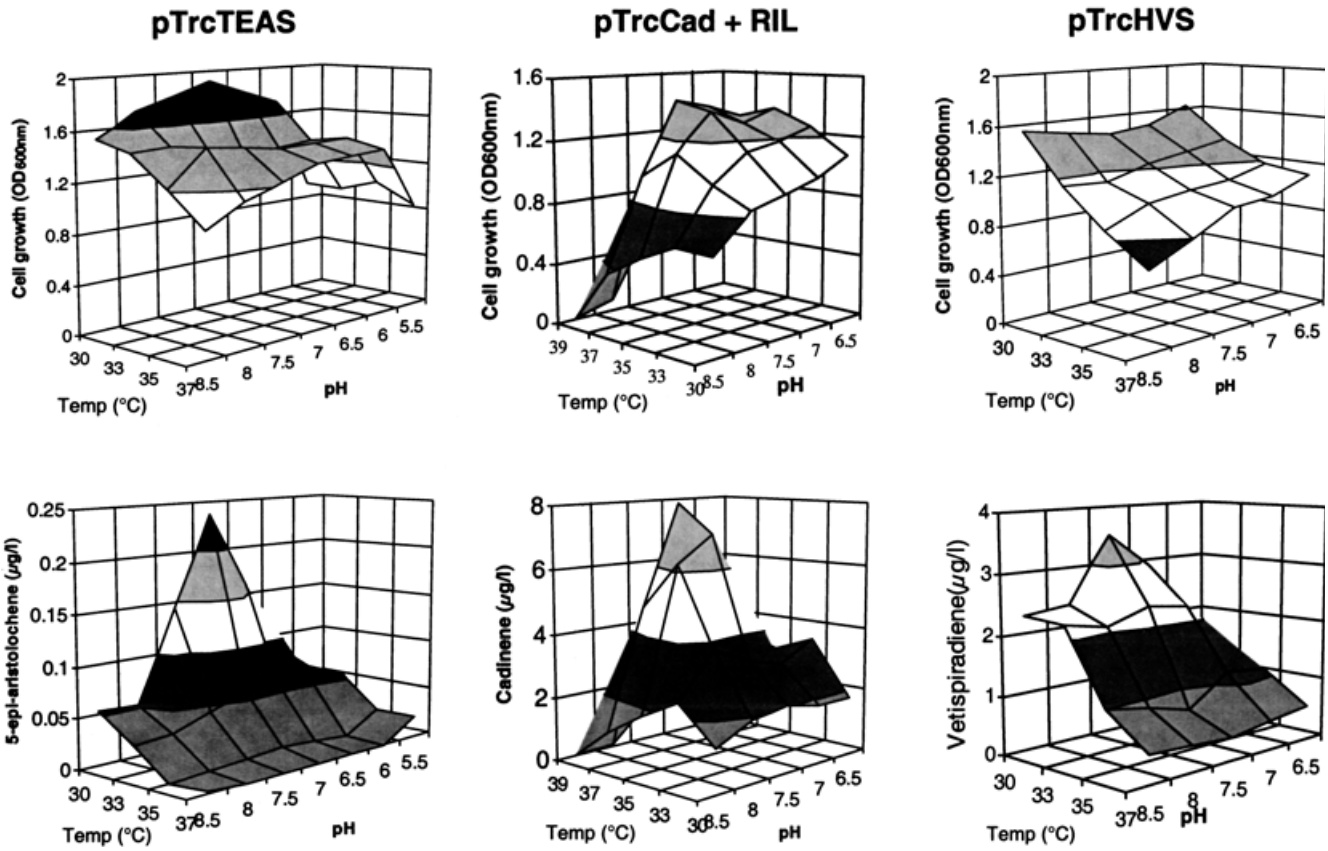

Figure 3. Optimization of pH and temperature conditions for 5-*epi*-aristolochene (pTrcTEAS), (+)-δ-cadinene (pTrcCad+RIL), and vetispiradiene (pTrcHVS) production in M9 medium supplemented with 0.2% glycerol. Concentrations of 5-*epi*-aristolochene, and vetispiradiene are reported as (+)-δ-cadinene equivalents. The temperature axis on the pTrcCad+RIL figures were reversed for clarity of the 3-D plots.

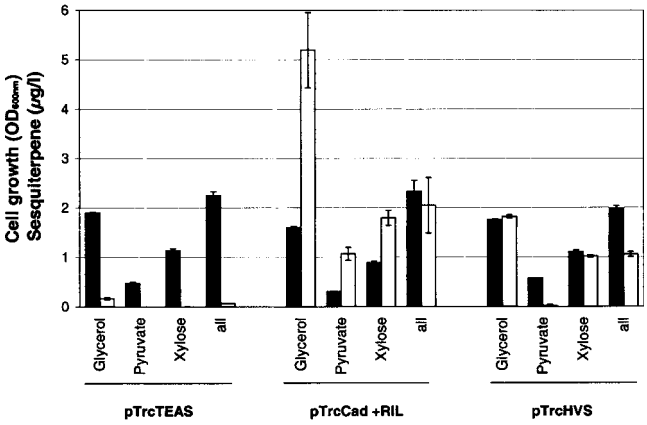

Figure 4. Comparison of the growth and sesquiterpene production of the *E. coli* clones expressing the cyclases in M9 medium supplemented with either 0.2% (v/v) of glycerol, pyruvate, xylose or all four carbon sources. Growth, black bars; sesquiterpene concentration, white bars. Concentrations of 5-*epi*-aristolochene and vetispiradiene are reported as (+)-δ-cadinene equivalents.

produced the lowest growth and yields (Fig. 4). Sesquiterpene production from pTrcTEAS fell below detection when using either pyruvate or xylose as carbon sources.

Sesquiterpene production by the *E. coli* strains HB101, DH10B, JM109, XL1-Blue, and DH5α was compared. DH5α was the best strain for the production of all three sesquiterpenes, although XL1-Blue was equally good for 5-*epi*-aristolochene synthesis (Fig. 5). Interestingly, the two strains DH5α and XL1-Blue were also the best hosts for production of lycopene, which also uses endogenous FDP as a precursor for synthesis (Kim and Keasling, 2001).

Time Course of Sesquiterpene Production and the Effect of Rare Codons on Sesquiterpene Production

We investigated the kinetics of sesquiterpene production to address three questions: (i) Are sesquiterpenes accumulated in the stationary phase? (ii) Are sesquiterpenes stable or volatile under our growth conditions? (iii) Can we improve sesquiterpene production in cells expressing the rare Arg, Ile and Leu tRNAs? The results show that sesquiterpenes are not accumulated in the stationary phase and that biosynthesis occurs mainly in actively growing cells (Fig. 6). Interestingly, 5-*epi*-aristolochene synthesis ceased in midlog phase, whereas (+)-δ-cadinene and vetispiradiene production continued up to but ceased in stationary phase (Fig. 6). The concentrations of 5-*epi*-aristolochene and vetispiradiene decreased slowly after cultures reached stationary phase, whereas the (+)-δ-cadinene concentration fell rapidly once the cultures reached maximum optical density. The volatility of (+)-δ-cadinene was tested by spiking the compound to a concentration of 0.1 μg/mL in 5 mL of sterile

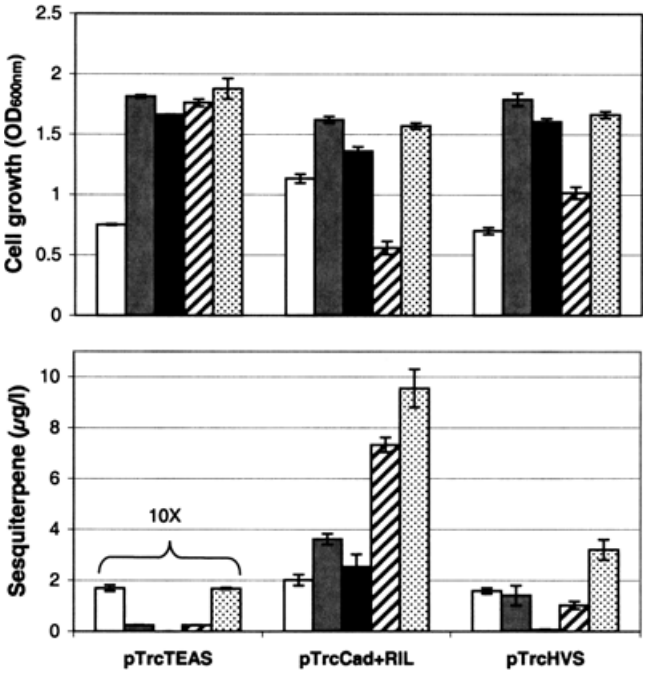

Figure 5. Comparison of *E. coli* host strains expressing sesquiterpene cyclases on growth in M9 + 0.2% (v/v) glycerol and sesquiterpene production. XL1-Blue, white bars; JM109, gray bars; HB101, black bars; DH10B, hashed bars; and DH5α, spotted bars. Concentrations of 5-*epi*-aristolochene and vetispiradiene are reported as (+)-δ-cadinene equivalents.

M9 medium prior to incubation at 37°C on a rotary shaker. (+)-δ-cadinene was not detected in culture medium after 8 hr of incubation. This indicated that the rapid decline in the sesquiterpene concentration after cultures reached stationary phase (Fig. 6) was likely due to loss of (+)-δ-cadinene by volatilization.

The effect of coexpressing the rare tRNAs along with the cyclase genes was different for all three enzymes. The growth of the pTrcTEAS clone and the production of 5-*epi*-aristolochene were not affected by coexpression of the RIL tRNA genes. However, the absolute production of (+)-δ-cadinene increased along with culture density in cells harboring the RIL plasmid, whereas approximately the same yields were maintained as the cells not harboring the RIL plasmid. In contrast, the growth rate of the pTrcHVS clone was reduced by the coexpression of the RIL plasmid, and vetispiradiene production decreased significantly in the absence of the RIL plasmid. Interestingly, both cultures attained a similar cell density after reaching stationary phase.

DISCUSSION

The recent discovery of a new pathway for IDP biosynthesis (Rohmer et al., 1993), and the rapidly accumulating wealth of information on expression and structure/function analysis of prenyltransferases (Wang and Ohnuma, 2000) and terpene cyclases (Starks et al.,

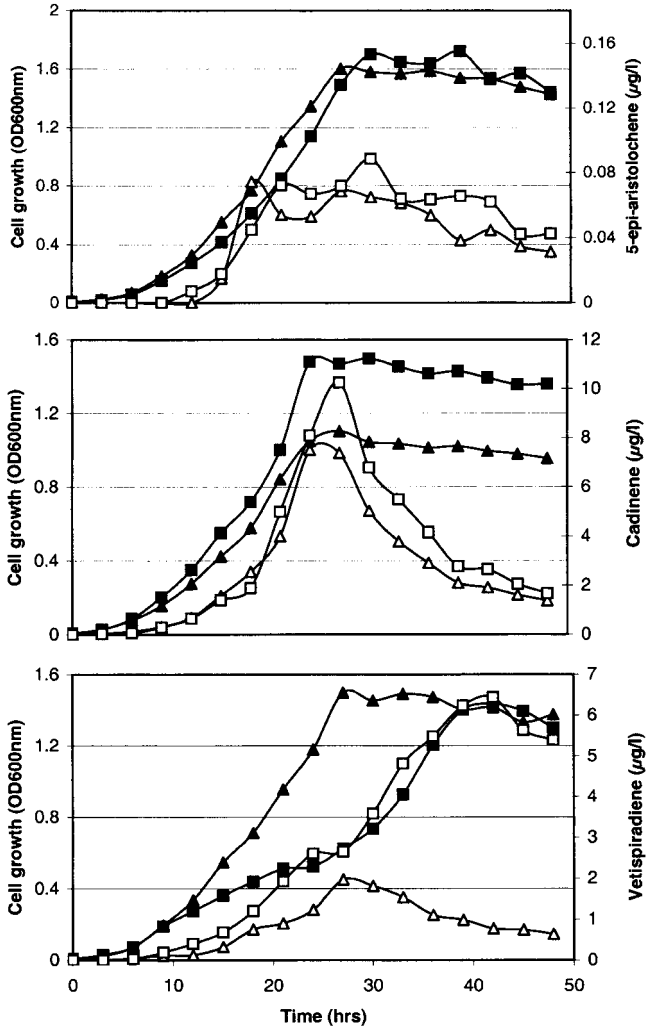

Figure 6. Growth (filled symbols) and sesquiterpene concentration (open symbols) as a function of time with strains harboring the rare codon plasmid RIL (squares) and strains without the RIL plasmid (triangles). Cultures of pTrcCad+RIL were incubated at 39°C. Concentrations of 5-*epi*-aristolochene and vetispiradiene are reported as (+)-δ-cadinene equivalents.

1997) have lead to a renewed interest in the isoprenoid biosynthetic pathways (Barkovich and Liao, 2001). As of 1998, more than 30 terpene cyclases were cloned from plants as cDNAs (Bohlmann et al., 1998), and the list is growing rapidly. These studies of plant terpene cyclases report principally on the cloning and characterization of the enzymes for substrate and product specificity, the mechanism of cyclization (Starks et al., 1997), and their expression profiles in plants (Mandujano-Chavez et al., 2000). In our study, we report on the heterologous production of three plant sesquiterpenes in *E. coli* with the objective of determining the best culture and expression conditions for their production.

Our first concern with expressing sesquiterpene cyclases in actively growing cultures was the potential toxicity of the intracellular production of sesquiterpenes on *E. coli*. We therefore compared growth rates and yields of IPTG-induced pTrc99A and sesquiterpene-expressing strains. The results showed that the growth of *E. coli* was not inhibited by the expression of the cyclases (data not shown). However, in a comparison with strains expressing the rare codon Arg, Ile, and Leu tRNAs (RIL plasmid), differences in growth rates for pTrcHVS and growth yields for pTrcCad were observed (Fig. 6). Vetispiradiene production by pTrcHVS increased from the coexpression of the RIL tRNAs, which suggests that expression of the enzyme was improved. However, the growth rate of the culture decreased, indicating that vetispiradiene may be toxic to *E. coli*. It should be noted that SDS-PAGE analysis of crude lysates of the strains expressing the cyclases showed no visible difference in protein bands of the predicted size (63 kDa) when compared with crude extracts of cells harboring pTrc99A (data not shown), indicating that the enzymes are poorly expressed under these conditions.

FDP production in *E. coli* is likely closely tied to growth because it is required for the biosynthesis of respiratory quinones and as lipid carriers in cell wall synthesis. Therefore, it was not surprising to see that (+)-δ-cadinene and vetispiradiene production closely followed exponential growth and ceased in stationary phase. Similar results were found for production of lycopene, which also uses FDP as the precursor for biosynthesis (Kajiwara et al., 1997). Others and we have previously shown that controlled overexpression of the 1-deoxy-D-xylulose-5 phosphate (DXP) synthase gene uncoupled carotenoid production from growth and enhanced lycopene in hosts engineered to synthesize lycopene (Harker and Bramley, 1999; Kim and Keasling, 2001). To determine if overexpression of DXP synthase would have the same effect on sesquiterpene production, we coexpressed the *E. coli dxs* gene along with the cyclase genes. No significant difference in sesquiterpene production was observed from the expression of the *dxs* gene (data not shown). By extrapolating carotenoid production values (10 mg/L at an OD_{600} of 6.0, Kim and Keasling, 2001) to (+)-δ-cadinene production values (10 μg/L at an OD_{600} of 1.5), we determined that the sesquiterpene production is ~500-fold lower than lycopene production. From this comparison, we could easily conclude that availability of the FDP precursor is not a limiting factor in sesquiterpene production. This would explain why overexpression of the DXP synthase had no effect on sesquiterpene yields and why the sesquiterpene cyclases scavenging the endogenous FDP did not affect the growth of the *E. coli* cultures.

Unlike (+)-δ-cadinene and vetispiradiene, 5-*epi*-aristolochene synthesis stopped in midexponential phase (Fig. 6). Back et al. (1994) reported that a rapid decline in TEAS protein and enzyme activity was observed 7 hr post-IPTG induction when the gene was expressed from the tac or T7 promoters. In addition, when 5-*epi*-aristolochene producing cultures were fractionated into cells

and supernatant and analyzed for sesquiterpenes, we observed that unlike (+)-δ-cadinene and vetispiradiene, 5-*epi*-aristolochene was only found in the cellular fraction (data not shown). Although we offer no evidence to support our hypothesis, we postulated that the instability of the protein and/or the inability of cells expressing TEAS to secrete 5-*epi*-aristolochene may have contributed to the sudden stop of sesquiterpene production in midexponential phase.

The data reported here demonstrates that a major limiting factor for the production of plant sesquiterpenes in *E. coli* is adequate expression of the enzymes and not flux to FDP. Furthermore, factors such as temperature, pH, choice of expression strain, and the presence of rare *E. coli* codons showed different effects on sesquiterpene production, which could not be predicted by cyclase gene sequence analysis alone. This information will become valuable for engineering plant sesquiterpene cyclases for high production levels in *E. coli* or other more suitable hosts.

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