Final Technical Report: DE-FG02-03ER15400, "Microbial Production of Isoprene"
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1. Summary of Progress

Isoprene is a volatile hydrocarbon of unknown function, produced by certain bacteria, plants and animals, sometimes in huge amounts—the Earth's forests are estimated to emit >500 x 10^6 tons of isoprene per year. With funding from this program we explored the biochemistry and regulation of isoprene formation in the model bacterial system, *Bacillus subtilis*, with the goals of explaining the biological rationale for isoprene biogenesis and constructing an isoprene-overproducing microbial system. Although the role for isoprene formation in *B. subtilis* is still uncertain, our current model for regulation of this hydrocarbon's synthesis is that isoprene production in *B. subtilis* is controlled by a combination of i) rapid regulation of isoprene synthase activity and ii) supply of the substrate for isoprene synthase, dimethyallyl diphosphate (DMAPP). This model parallels our current thinking about the control of isoprene formation in plant chloroplasts. In this reporting period we have been working to test part ii) of this model; this work has produced new results using genetic and analytical approaches. For examples, we have developed an analytical method to resolve DMAPP and its isomer, isopentenyl diphosphate, from each other in bacteria and plants. We have also shown that the IPP isomerase (type 2) of *B. subtilis* is not the source of "isoprene synthase" activity, and discovered that *B. subtilis* releases C5 isoprenoid alcohols to the medium, suggesting that isoprene plus other C5 isoprenoids may be common by-products of metabolism. In addition, we have continued to work on our discovery that wild type *B. subtilis* strains form prolific biofilms, are normal components of plant root microflora, and are testing the idea that *B. subtilis* growing in biofilms uses isoprene to induce plant root exudation. Progress in these areas is briefly summarized below.

A) Manipulations of the deoxyxylulose phosphate (DOXP) pathway and the effects of such manipulations on isoprene production. Isoprenoids in *B. subtilis* are synthesized via the deoxyxylulose phosphate (DOXP) pathway, which gives rise to the C5 building blocks of all isoprenoids, IPP and DMAPP (Scheme 1 below). We have been working to alter the carbon flow of the DOXP pathway to either elevate or diminish cellular DMAPP levels, and determine if the control of isoprene formation is driven by substrate supply. Scheme 1 shows the points at which we attempted to silence *B. subtilis* genes, namely IPP isomerase (*ypgA*), which interconverts IPP and DMAPP, the substrate for isoprene production, and undecaprenyl pyrophosphate synthetase (*uppS*), which catalyzes a reaction producing higher isoprenoids. The *ypgA* null mutant has been isolated and confirmed by PCR and DNA sequencing of genomic DNA; similar analyses of the *uppS* null mutant were not conclusive. We expected that the isomerase mutant would have lower DMAPP levels and the synthetase mutant will have elevated DMAPP levels, but only the data for the isomerase mutant is in hand. Surprisingly, i) the isomerase mutant had higher DMAPP levels (measured by the HPLC method described below), and ii) when run in a bioreactor, the IPP isomerase mutant produced the characteristic three peaks of isoprene release seen from the parent strain, and when grown in minimal medium grows better than the parent strain. Clearly, the IPP isomerase is not needed for growth in the rich or minimal media we use, but its role, if any, in controlling DMAPP levels may be revealed in other types of experiments.

In the course of manipulating DOXP pathway genes, we have also isolated two isoprene null mutants, probably by some type of "illegitimate" recombination. The location of insertion of the antibiotic resistance marker in the *B. subtilis* chromosome is a subject of future investigation, as

we want to identify which gene disruption(s) eliminate isoprene formation. This information could be very important to understanding the role of isoprene formation in these bacteria.

B) The IPP isomerase (type 2) of *B. subtilis* is not the source of "isoprene synthase" activity. All organisms interconvert IPP and DMAPP during isoprenoid biosynthesis using IPP isomerases. It has recently been discovered that *B. subtilis* has a type II IPP isomerase; this class of IPP isomerases requires a reduced flavin cofactor, and appears to be more common in organisms (or chloroplasts) using the DOXP pathway rather than the mevalonate pathway. Since the type II IPP isomerases use a different mechanism than the type I enzymes, we postulated that they might produce isoprene as a by-product. However, as mentioned in A) the Δ*ypgA* null mutant produces isoprene in a pattern identical to the parent strain, demonstrating that the type II IPP isomerase is not the source of "isoprene synthase" activity.

C) A new quantitative method for analysis of cellular pools of DMAPP and IPP. In the past, with funding from this DOE grant, we developed and published a sensitive method for measuring cellular DMAPP levels. Now, we have developed a method to allow for simultaneous, quantitative analysis of both DMAPP and its isomer IPP by high resolution HPLC. We applied a method for the separation of oligonucleotides, and adapted it to the full separation of DMAPP and IPP. The cellular levels of these two isomers, which are interchanged by the IPP isomerases mentioned above, may be critical to the controls on isoprene formation in both bacterial cells and plant chloroplasts. We have obtained the first quantitative data for DMAPP and IPP in bacterial and plant cells, under different growth or physiological conditions, and are preparing a manuscript for publication. As mentioned above, this method was used to test the substrate control theory for isoprene formation.

D) Release of isoprenoid alcohols during *B. subtilis* growth. We have discovered that during normal growth *B. subtilis* releases a compound or compounds that can be converted to isoprene in strong acid. Fractionation of the medium suggests that this compound(s) is not DMAPP, but is one of the methylbutenols, known to be converted to isoprene in acid. HPLC analysis currently suggests that the main compound is 3-methyl-2-buten-1-ol and that 3-methyl-3-buten-1-ol is also present in significant amounts. GC-MS analysis will be used for confirmation. The likely source of these alcohols would be DMAPP and IPP, respectively, resulting from cellular pyrophosphatase activity (Scheme 1). It is not clear why cells would produce these hydrolytic products, unless there is a build-up of cellular IPP and DMAPP, perhaps under conditions when higher isoprenoid levels are sufficient for growth. We worked to quantify the release of these alcohols during *B. subtilis* growth and relate their formation to both isoprene release and cellular DMAPP and IPP levels, but were not able to complete this task with suitable replicates.

E) Root colonization and biofilm formation in wild type *B. subtilis*. During studies of the nutritional control of isoprene formation in *B. subtilis*, we accidentally discovered that wild type strains isolated from plant roots, but not domesticated laboratory strains, can form extensive biofilms. We found that the most prolific biofilm-forming isolates also produced isoprene, leading to the idea that isoprene might be a signaling or quorum sensing molecule. Biofilm formation was also shown to be highly dependent on the presence of extracellular potassium ion and the lipopeptide, surfactin. Using transposon mutagenesis we have isolated a variety of mutants defective in biofilm formation, and tested each for isoprene production. Isoprene formation, however, was not greatly affected in these mutants. A manuscript describing these mutants has been published.

We do not consider this approach a dead end, however. We have developed an alternate theory that isoprene might be a volatile signaling molecule in the rhizosphere, the normal habitat of *B. subtilis*, perhaps eliciting root exudation of nutrients. With the help of a collaborator, we are planning to test the wild type strain, our biofilm mutants, and our isoprene-null mutants for elicitation of root exudation in cultured Arabidopsis roots. The model we propose is that both biofilm attachment and isoprene release may be essential for elicitation of exudate release. We have also published a paper on the facile isolation of *B. subtilis* and related species from plant roots.

F) Control of isoprene formation in plant leaves. As with *B. subtilis*, the control of isoprene formation in plant chloroplasts is uncertain. In order to see if the DMAPP overflow model for isoprene formation is common to bacteria and plants, we used cottonwoods to determine if alteration of the flow of carbon precurors such as phosphoenolpyruvate (PEP) into the DOXP pathway would alter DMAPP levels and isoprene emission rate. The results show that this is the case. Elevation of PEP carboxylase, induced by growth of plants with nitrate as sole N source, led to induction of PEP carboxylase and a decrease in both DMAPP and isoprene formation. These published results show that controls on isoprene formation may be very similar in both bacteria and chloroplasts.

G) Publications citing support from this project (2003-2006)

T.G. Custer, W.P. Wagner, S. Kato, V.M. Bierbaum, and R. Fall (2003) Potential of on-line CIMS for bioreactor monitoring, *Biotechnol. Prog*., 19, 1355-1364. (This paper represents completion of bioreactor experiments from 2002, including on-line identification of isoprene and numerous other volatile metabolites).

R.F. Kinsinger, M.C. Shirk, and R. Fall (2003) Rapid surface motility and biofilm formation in *Bacillus subtilis* is dependent on extracellular surfactin and potassium ion. *J. Bacteriol*., 185, 5627-5631

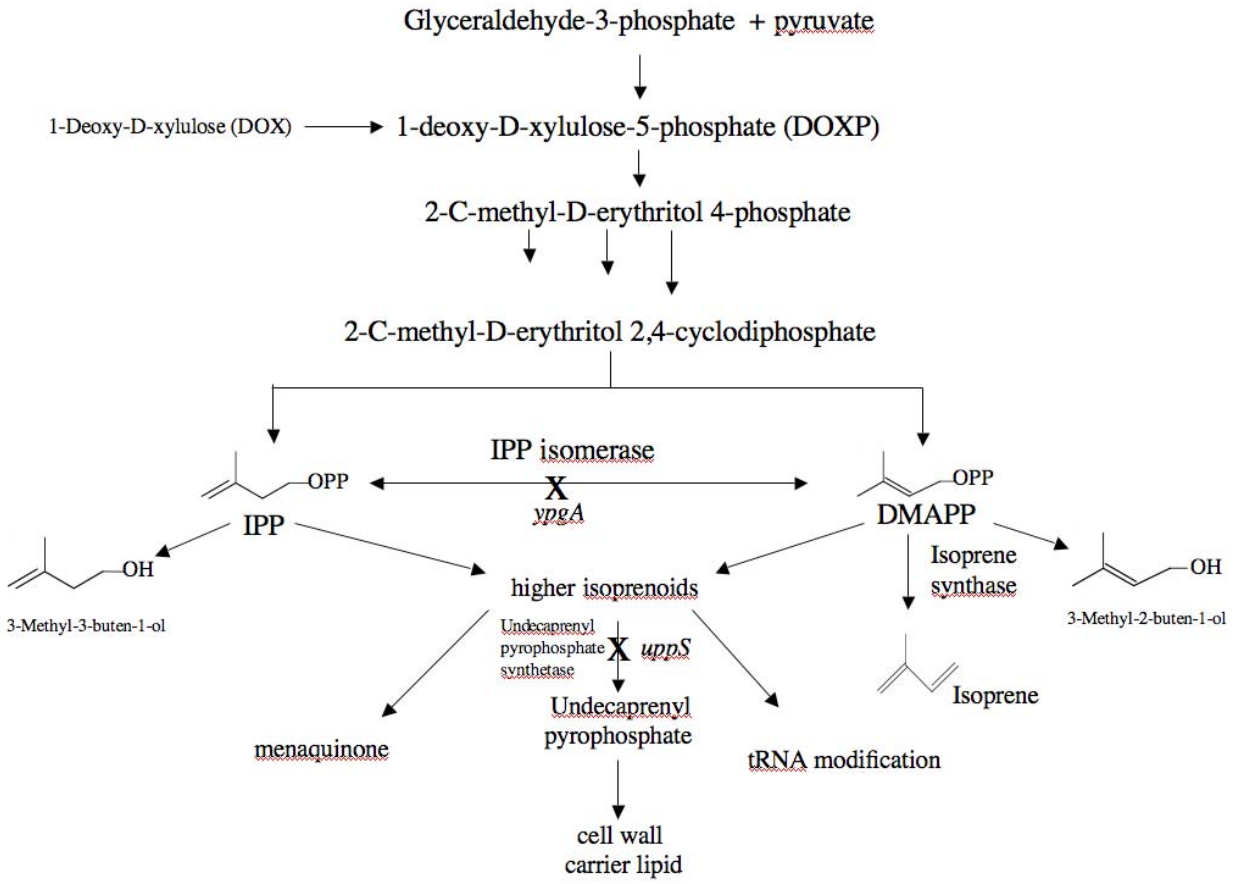
H.P. Bais, R. Fall, and J.M. Vivanco (2004) Biocontrol of *Bacillus subtilis* against infection of Arabidopsis roots by *Pseudomonas syringae* is facilitated by biofilm formation and surfactin production, *Plant Physiol*.,134, 307-319.

T.N. Rosenstiel, A.L. Ebbets, W.C. Khatri, R. Fall, and R.K. Monson (2004) Induction of poplar leaf nitrate reductase: a test of extrachloroplastic control of isoprene emission rate, *Plant Biol.*, 6, 12-21.

R. Fall, R.F. Kinsinger, and K.A. Wheeler (2004) A simple method to isolate biofilm-forming *Bacillus subtilis* and related species from plant roots. *Systematic and Applied Microbiology*, 27, 372-379.

R.F. Kinsinger, D.B. Kearns, M. Hale, and R. Fall (2005) Genetic requirements for potassium ion-dependent colony spreading in *Bacillus subtilis*. *Journal of Bacteriology*, 187, 8462-8469.

T. Sivy, C. Calahane, and R. Fall. A new, quantitative method for determination of cellular prenyl diphosphates. In preparation.



Scheme 1. The DOXP pathway and isoprenoid biosynthesis in *Bacillus subtilis*. Some of the genes of interest are indicated, and those that we have disrupted by insertional mutagenesis are indicated with an "X." The disruption of the *uppS* gene could not be verified by PCR.