Final Technical Report: DE-FG03-97ER20274, "Microbial Production of Isoprene"
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1. Summary of Progress

We have discovered that microorganisms produce and emit the hydrocarbon isoprene (2-methyl-1,3-butadiene), and have suggested that if isoprene-producing enzymes and their genes can be harnessed, useful hydrocarbon-producing systems might be constructed. The main goal of the proposed work was to establish the biochemical mechanism and regulation of isoprene formation in the model bacterial system, *Bacillus subtilis.* Specific objectives of the proposed work were the following: (a) to characterize the physiological regulation of isoprene formation in *B. subtilis*; (b) to characterize mutations in *B. subtilis* 168 that suppress isoprene formation, clone these genes, and determine how isoprene and isoprenoid carbon flow are regulated; and (c) to test "overflow" and "signaling" models for *Bacillus* isoprene formation. We also pursued exploratory studies with *Bacillus* to determine if these bacteria could serve as a model for isoprene formation in plant chloroplasts, and to determine if these isoprene-forming bacteria are associated with plant roots (where isoprene release might serve a signaling function in the rhizosphere). The following sections highlight our progress in this research.

1.1. The biochemical basis of isoprene formation in *B. subtilis*. We worked steadily to characterize the enzyme responsible for isoprene synthesis in *B. subtilis,* and this proved to be extremely challenging. It was only possible to obtain active enzyme at certain growth stages and only if high levels of phosphatase inhibitors were added. We were able to assay the enzyme in permeabilized cells, and show throughout *B. subtilis* growth in a bioreactor that the enzyme rises and falls with each of three peaks of isoprene release (i.e. it appears to be a regulated enzyme). Our model for isoprene regulation, as yet unproven, is that isoprene synthase activity is controlled by phophorylation/dephosphorylation. These results and initial characterization of the enzyme have been published. It is notable that analysis of the *B. subtilis* genome shows no gene sequence that is analogous to the cloned plant (poplar) isoprene synthase.

1.2. Mutations that control isoprene formation. We explored genetic aspects of isoprene formation in *B. subtilis*. Mutant characterization focused on analysis of aerobic fermentation experiments, where *B. subtilis* 6051, the wild-type Marburg strain, exhibits three phases of isoprene formation during the course of growth on a standard glucose-tryptone medium. An interesting finding is that when the domesticated *B. subtilis* strain 168, used for the *Bacillus* genome project, is grown under identical conditions, only phase 1 isoprene is formed. *B. subtilis* 168 was derived from strain 6051 by radiation mutagenesis, and has been presumed to harbor only the trpC2 mutation. We constructed a ΔtrpC deletion strain in the 6051 genetic background, and found that its isoprene formation pattern is essentially the same as the parent. In addition, transformation of stain 168 with 6051 DNA allowed isolation of a trp prototroph with restored isoprene formation. Our results suggest that strain 168 carries additional mutations that suppress isoprene formation in phase 2 and 3, and that transformation at the trpC locus also affected nearby genes during recombination.

1.3. Testing "overflow" and "signaling" models for *Bacillus* isoprene formation. Earlier, we had formulated two models to explain why isoprene is formed in Bacillus: 1) an isoprenoid overflow model, and 2) a signaling model. The isoprenoid overflow model proposes that general cell growth and expression of particular catabolic pathways requires the formation of specific isoprenoids, and that when these isoprenoids are not being synthesized carbon from dimethylallyl diphosphate (DMAPP) overflows to isoprene. Thus, isoprene synthesis would act as a metabolic "safety valve." A second model for *Bacillus* isoprene formation is that the hydrocarbon acts as a volatile signaling molecule or is part of a signaling pathway. For example, cells in nutrient sufficient conditions might make a repressor of stationary phase or sporulation genes. Isoprene could be such a repressor, or isoprene could serve as a signal for carbon availability in *Bacillus* biofilms (see 1.5).

We obtained supportive evidence for the second model as follows. We focused on the metabolic linkage between acetoin biosynthesis/catabolism and isoprene formation. Using gene silencing methods, we constructed deletions in acetolactate synthase (alsS), which blocks acetoin biosynthesis, and acoA (a component of acetoin dehydrogenase), which blocks acetoin catabolism. These mutants have been analyzed in aerobic growth experiments, revealing substantial pleiotropic effects on isoprene formation and other cellular processes. For example, the ΔalsS mutant loses pH control, shows no phase 2 or 3 isoprene, and does not sporulate. These properties can be restored by controlling growth pH. The ΔacoA mutant is unable to catabolize acetoin released into the medium, and as a result the formation of phase 2 and 3 isoprene is abnormal. Additional findings show that phase 3 isoprene is probably related to amino acid catabolism during the late stages of growth, consistent with a role for isoprene as a product or signal arising during the rapid catabolism of available carbon sources. We have formulated a "carbon barometer" model to explain the role of isoprene in sensing carbon availability. This work has been published.

1.4. Is *Bacillus* isoprene formation analogous to chloroplast processes? This research was initiated because of the continuing interest in the puzzle of isoprene formation in leaf chloroplasts. As with *Bacillus*, it is not known why some plants release such a large fraction of photosynthetic carbon as isoprene. In pursuit of linkages between bacterial and plant isoprene formation, we used our DMAPP assay to demonstrate that leaves of the isoprene-emitter (cottonwood) show a diurnal cycle, peaking at mid-day in parallel with isoprene release. Thus it appears that in two different systems controls on isoprene formation might be regulated, and linked to isoprenoid carbon availability. This work has been published.

Supporting results were obtained by $^{13}CO_2$ labeling of leaf isoprene. For the first time we used on-line proton transfer reaction mass spectrometry to observe the kinetics of ^{13}C incorporation into emitted isoprene. We were able to establish the lifetimes of individually ^{13}C-labeled isoprene species, including the fully ^{13}C-labeled species, and demonstrate that there is a source of extrachloroplastic carbon that bypasses the DOXP pathway to produce DMAPP. The data obtained shed light on the half-lives of photosynthetic metabolites, exchanges of carbon between cellular pools, and suggest multiple origins of isoprene precursors in leaves. This work has been published.

1.5. On-line analysis of isoprene and other volatile metabolites in bioreactors. Our analysis of rapidly changing isoprene formation in bioreactors has raised the need for an on-line method of isoprene analysis, that is, an analytical tool that would constantly monitor isoprene emerging

from a bioreactor. It would be even more useful if such an analytical tool could monitor other bacterial volatile metabolites that are diagnostic of the metabolic state of the cells. With this DOE funding and a University seed money grant, we were able to analyze *Bacillus* volatiles on-line with a proton-transfer-reaction chemical ionization mass spectrometer (PT-CIMS). Our bioreactor was co-located with the PT-CIMS instrument, we demonstrated the feasibility of continuous measurements of isoprene, and numerous *Bacillus* volatiles (acetaldehyde, diacetyl, acetoin, ethanol, acetone, butanol, etc.). This work has been published, and this technology will be very valuable in our future work to monitor metabolic engineering of isoprenoid biosynthesis.

1.6. Plant roots are a source of biofilm-forming *B. subtilis* and *B. subtilis* can provide biocontrol of *Pseudomonas syringae* on plant roots. We wanted to compare isoprene formation in wild-type *B. subtilis* isolate*s*, not just rely on culture collection strains. Based on the *B. subtilis* genome, there are hints that this bacterium is associated with plant roots. We discovered that roots of a variety of plants (sugar beet, carrot, radish, corn) contain tightly bound *Bacillus* sp. These bacteria are probably not derived from adhering soil, since they are not easily removed by repeated sonic rinsing. Of particular interest is the fact that these bacteria form profuse, rapidly spreading biofilms on media that we have developed. Over 40 isolates have been obtained from different plants in this way. Traditional phenotypic tests show that all are aerobic, gram-positive, spore-formers, and 16S RNA analysis identified isolates as *B. subtilis* and its close relatives. All produce isoprene in similar amounts in liquid media, but detailed bioreactor experiments have not yet been completed. In addition, the link between biofilm growth and isoprene release is under study, and initial results suggest that the highest levels of isoprene formation on solid media occurs during maximal biofilm growth. This is consistent with a role for isoprene as an intercellular signal for quorum sensing of carbon availability. Many of these isolates also form antifungal compounds, and appear related to commercial seed-treatment strains of *Bacillus*.

Using *B. subtilis* 6051 we were able to demonstrate that stable biofilms can be formed on cultured Arabidopsis roots, and that such treated roots are highly resistant to killing by a plant pathogenic *Pseudomonas syringae*. This biocontrol was not seen with a biofilm-negative mutant that we constructed; this mutant contained a disruption of a surfactin synthesis gene, so it appears that formation of *B. subtilis* biofilms on plant roots is dependent on the release of surface-active surfactin peptides, which serve both to facilitate adherence to roots and also to kill *P. syringae*. This work is in press. The role of isoprene formation as a volatile signal between *B. subtilis*, roots and/or *P. syringae* is still under investigation.

During the course of this work we also demonstrated that rapid surface motility and biofilm formation in *Bacillus subtilis* is dependent on formation of extracellular surfactin and potassium ion, and these observations have been published.

2. Publications (arising from this 3 year grant)

A.J. Fisher, T.N. Rosenstiel, M.C. Shirk, and R. Fall (2001) A Non-radioactive assay for cellular dimethylallyl diphosphate. *Analyt. Biochem.*, 292, 272-279.

T. Sivy, M.C. Shirk, and R. Fall (2002) *Bacillus subtilis* isoprene synthase activity parallels fluctuations in isoprene release during growth. *Biochem. Biophys. Res. Commun*., 294, 71-75.

T.D. Rosenstiel, A.J. Fisher, R. Fall, R.K. Monson (2002) Differential accumulation of dimethyallyl diphosphate in leaves and needles of isoprene-emitting, methylbutenol-emitting and non-emitting species. *Plant Physiol*., 129, 1276-1284.

T. Karl, R. Fall, T. Rosenstiel, P. Prazeller, M. Duane, G. Seufert, and W. Lindinger (2002) On-line analysis of the $^{13}CO_2$ labeling of leaf isoprene suggests multiple subcellular origins of isoprene precursors. *Planta*, 215, 894-905.

M.C. Shirk, W.P. Wagner and R. Fall (2002) Isoprene formation in *Bacillus subtilis*: A barometer of central carbon assimilation in a bioreactor? *Biotechnol. Prog*., 18, 1109-1115.

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.

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. Pal Bais, R. Fall, and J. Vivanco (2003) Biocontrol of *Bacillus subtilis* against infection of *Arabidopsis thaliana* roots by *Pseudomonas syringae* is facilitated by biofilm formation and surfactin production, *Plant Physiol*., in press.

R. Fall, R.F. Kinsinger, and K.A. Wheeler (2003) A simple method to isolate biofilm-forming *Bacillus subtilis* and related species from plant roots. System. Appl. Microbiol., under review.