Progress Report: DE-FG03-97ER20274, "Microbial Production of Isoprene"
Principal Investigator: Ray Fall, University of Colorado
Dates Covered: 6/15/01 to 3/1/02

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 is to establish the biochemical mechanism and regulation of isoprene formation in the bacterial system, *Bacillus subtilis*. Specific objectives of the proposed work are 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 are also pursuing the isolation and cloning of *B. subtilis* isoprene synthase, which we believe may be a regulatory enzyme.

(A) The biochemical basis of isoprene formation in *B. subtilis*. We have been working steadily for the past year to characterize the enzyme responsible for isoprene synthesis in *B. subtilis*. This has proven to be extremely challenging. As we reported last year, it is only possible to obtain active enzyme at certain growth stages and only if high levels of phosphatase inhibitors are added. A graduate student, Tami Sivy, has now been able to assay the enzyme in permeabilized cells, and shown 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). It is possible that isoprene synthase activity is controlled by phophorylation and dephosphorylation. Tami is now developing a rapid purification scheme for the enzyme, based on the idea that one has to quickly separate the phosphorylated enzyme from a protein phosphatase that inactivates it. She has begun testing purified enzyme fractions with using proteomic methods (i..e. MALDI-MS analysis of peptide digests of protein bands on SDS-PAGE gels). From the published *B. subtilis* genome we expect to obtain the isoprene synthase gene sequence and chromosome location soon. This will allow us to determine if isoprene synthase is part of a known operon, to clone and overexpress the enzyme for detailed characterization, and prepare to manipulate the level of isoprene synthase gene expression.

(B) Mutations that control isoprene formation. We have continued to explore genetic aspects of isoprene formation in *B. subtilis*. Mutant characterization has 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 widely used *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 have constructed a ? trpC 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 strain 168 with 6051 DNA allowed isolation of a trp prototroph with restored phase 2 and 3 isoprene formation. Our results suggest that strain 168 carries additional mutations that suppress isoprene formation, and that these genes may lie close to the trpC locus. Isoprenoid synthetic genes are also located near trpC, suggesting a link between mutations in this region and general isoprenoid biosynthesis. The role of these genes in isoprene formation is under investigation.

(C) Testing "overflow" and "signaling" models for *Bacillus* isoprene formation. Earlier, we had formulated two models to explain why isoprene is formed in bacilli: 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 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 F).

We have recently obtained supportive evidence for the first model as follows. We have been able to measure the levels of cellular DMAPP (see (D) below) and isoprene synthase activity in growing *B. subtilis* during phase 1 of isoprene release. Both cellular level of DMAPP and the activity of isoprene synthase increase and decrease rapidly in parallel with isoprene release from the cells. These results argue that the isoprene biosynthetic pathway is controlled by central carbon availability. We are planning to extend our analyses to other key enzymes of the isoprenoid pathway, and relate lack of isoprene formation in strain 168 to changes in cellular DMAPP and isoprene synthase. We are also independently testing whether isoprene is a repressor of late genes for competence, flagella, protease, sporulation, etc.

(D) Is *Bacillus* isoprene formation analogous to chloroplast processes? This research program 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 biological systems controls on isoprene formation might be regulated, and linked to isoprenoid carbon availability. Support for further work on the cottonwood system is being requested from another funding source, but continued parallel work in our laboratory on both bacterial and plant systems may foster new ideas on construction of recombinant systems that overexpress isoprene or other isoprenoids.

(E) 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 instrument 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 have undertaken preliminary attempts 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, and several data sets from growth of *B. subtilis* have been collected. Although excess water in the air stream from the bioreactor complicates the analysis, this work has demonstrated the feasibility of continuous measurements of isoprene, and numerous *Bacillus* volatiles (acetaldehyde, diacetyl, acetoin, ethanol, acetone, butanol, etc.). This technology will be very valuable in our future work to monitor isoprenoid biosynthesis in *Bacillus* strains.

(F) Plant roots are a source of biofilm-forming *B. subtilis*; 16S RNA identification. We wanted to examine isoprene formation in wild-type *B. subtilis* isolates, 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 have 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. 16S RNA analysis has been established using a combination of (i) universal bacterial 16S RNA primer and (ii) a specific *B. subtilis* 16S RNA primer. Sequencing of the resulting amplified DNAs show that of the first 8 isolates tested, 4 are 100% identical with *B. subtilis*, 3 are >98% identical with *B. subtilis*, and 1 is *B. licheniformis*. All produce isoprene in similar amounts, but detailed bioreactor experiments have not yet been completed. In addition, the link between biofilm growth and isoprene release is under study to see if the hydrocarbon could be some type of 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*.

(G) Publications from this project

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

T.N. Rosenstiel, A.J. Fisher, R. Fall, and R.K. Monson (2002) Differential accumulation of dimethylallyl diphosphate in leaves and needles of isoprene-emitting, methylbutenol-emitting, and non-emitting species. *Plant Physiology*, in press.

T. Sivy, M.C. Shirk, and R. Fall (2002) *Bacillus subtilis* isoprene synthase activity parallels fluctuations in isoprene release during growth. Submitted to *Biochemical Biophysical Research Communications.*

M.C. Shirk, W.P. Wagner, and R. Fall (2002) Isoprene formation in *Bacillus subtilis*: a barometer of central carbon metabolism in a bioreactor. Submitted to *Biotechnology Prog*ress

T.G. Custer, W. Wagner, S. Kato, V.M. Bierbaum, and R. Fall (2002) On-line analysis of volatile metabolites in bacterial bioreactors using CIMS. In preparation for *Biotechnology Prog*ress

2. Work to be Performed

We will continue to address the major goals described above. The main emphasis will be on cloning and overexpressing *B. subtilis* isoprene synthase. This will allow us to complete the biochemical characterization of the enzyme, establish the mechanism of regulation of the enzyme, and locate the gene in the *Bacillus* genome (possibly in a known operon). Using gene disruption methods that we have established, we will be able to knock out the isoprene synthase gene and establish if it is essential. Work is planned to establish how both isoprene synthase and levels of its substrate (DMAPP) are regulated during growth, possibly as part of a carbon assimilation sensing/signaling system. We will also continue work on isoprene formation in *B. subtilis* biofilms, which are abundant on root surfaces, addressing the idea that isoprene could be a quorum sensing molecule during biofilm growth. If evidence for a signaling role is established, exploratory work will address whether there is a cellular receptor of isoprene. This work is

continuing to progress rapidly towards establishing, for the first time, why any biological system produces isoprene. The information gained will be related to light-regulated isoprene and DMAPP biochemistry in plant chloroplasts, information that might aid the design of bio-systems that overproduces isoprene or other isoprenoids.

3. Estimated Unobligated Funds: less than 10%.

4. Proposed Budget for the next year: same as award letter ($95,000)