Isoprene Biosynthesis in *Bacillus subtilis* via the Methylerythritol Phosphate Pathway

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Isoprene (2-methyl-1,3-butadiene), an abundant natural product of unknown function in plants, has recently been found to be one of the major volatiles formed by *Bacillus subtilis*. To understand the metabolic origins of isoprene in *B. subtilis*, we used ^{13}C- and ^{2}H-labeling methods with GC−MS analysis of released isoprene. The results indicate that, in this bacterium, isoprene is not formed by the mevalonate pathway or from catabolism of leucine, but, as in plant systems, it is a product of the methylerythritol phosphate pathway of isoprenoid synthesis. This work supports the idea that *B. subtilis* could be used as a microbial model for studying the biochemistry of isoprene formation.

Isoprene (2-methyl-1,3-butadiene) is one of the most abundant natural products formed in the biosphere. Green plants are estimated to produce and release about 500 million tons of isoprene to the atmosphere annually;[1] yet, despite decades of research on isoprene formation in plants and progress in understanding the enzymatic basis of isoprene synthesis in chloroplasts,[2−4] the metabolic rationale for its formation has remained elusive.[5,6] Our recent discovery of isoprene formation in the Gram-positive bacterium *Bacillus subtilis*[7,8] has provided the opportunity to study isoprene biogenesis in a microbial system that is highly amenable to genetic, molecular biologic, and genomic approaches.[9]

Until recently, the universal pathway of isoprenoid biosynthesis in living organisms was thought to involve the mevalonate pathway.[10] However, numerous labeling studies in a wide range of plants and microorganisms have now shown that a different pathway for the early steps of isoprenoid biosynthesis, the methylerythritol phosphate (MEP) pathway, operates in some Gram-negative bacteria and plant chloroplasts.[10−12] In the MEP pathway, isoprenoids are derived from condensation of pyruvate and glyceraldehyde-3-phosphate to give 1-deoxy-D-xylulose-5-phosphate, which is reduced and isomerized to form 2-*C*-methyl-D-erythritol 4-phosphate (MEP). MEP can then be converted to isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), the building blocks of isoprenoid biosynthesis.

Isoprene is the simplest isoprenoid formed in *Bacillus*, and it seems likely that it is produced from DMAPP by a pyrophosphate elimination reaction, as occurs in plants.[2,3] This has not yet been established, however, and it is also possible that in *Bacillus* the 2-methylbutane carbon skeleton of isoprene is derived from IPP or from some other precursor. Here we attempt to understand the metabolic origins of isoprene in *B. subtilis*, using ^{13}C- and ^{2}H-labeling, information obtained with a specific inhibitor of the mevalonate pathway, and from genomic analysis.

Results and Discussion

We employed ^{13}C-labeling experiments to obtain evidence for the presence of the mevalonate or MEP pathways in *Bacillus*. *B. subtilis* 6051, a wild-type strain, was grown on various ^{13}C substrates, and the isoprene released into the headspace was analyzed by GC−MS with a custom-made gas preconcentration inlet system (see below). During electron impact ionization mass spectrometry, unlabeled isoprene (molecular mass 68) fragments into three major ions with mass-per-charge ratios (*m*/*z*) of 67, 53, and 39 resulting from hydrogen loss or breakage of either C−C single bond. When *B. subtilis* 6051 cultures were grown in an F salts−tryptone medium containing unlabeled glucose ([^{12}C]glucose), the mass spectrum of emitted isoprene exhibited these three main fragments. The isoprene measured was produced in the first phase of isoprene formation, which corresponds to glucose catabolism.[13] A parallel experiment with U-[$^{13}C_6$]glucose produced isoprene, with the GC−MS fragmentation pattern showing the main isoprene fragments at *m*/*z* 72, 57, and 42 amu. Table 1 summarizes the measured abundances of the three main fragments of isoprene seen in these experiments; for simplicity, the data for unlabeled fragments (i.e., 67, 53, and 39 amu) are omitted because they represented less than 5% of detected ion fragments. These shifts in ion abundance for all three major isoprene ion fragments clearly indicate full ^{13}C labeling of isoprene. For example, mass ion 67 (derived from [^{12}C]glucose) was shifted to mass ion 72 with U-[$^{13}C_6$]glucose as the substrate, indicating that all five isoprene carbons contain ^{13}C isotopes. The labeling also indicates that under these growth conditions all of the isoprene was derived from glucose carbons, rather than

Table 1. Ion Signals for the Three Major Mass Spectral Fragments of Isoprene Produced by *B. subtilis* 6051 Using Various Labeled-Carbon Sources

	isoprene fragments, *m*/*z*, (relative abundance)[a]		
substrate	fragment 1	fragment 2	fragment 3
[^{12}C]glucose	39 (57)	53 (52)	67 (100)
U-[$^{13}C_6$]glucose	42 (45)	57 (53)	72 (100)
[^{12}C]pyruvate	39 (53)	53 (54)	67 (100)
1-[^{13}C]pyruvate	40 (86)	54 (35)	68 (100)
2-[^{13}C]pyruvate	40 (98)	55 (66)	69 (100)
3-[^{13}C]pyruvate	40 (37)	54 (46)	69 (100)
[^{1}H]leucine	39 (39)	53 (64)	67 (100)
5,5,5-[$^{2}H_3$]leucine	39 (58)	53 (73)	67 (100)
isopropyl-[$^{2}H_7$]leucine	39 (44)	53 (55)	67 (100)

[a] Typical data from one experiment; similar results were seen in a second, independent experiment.

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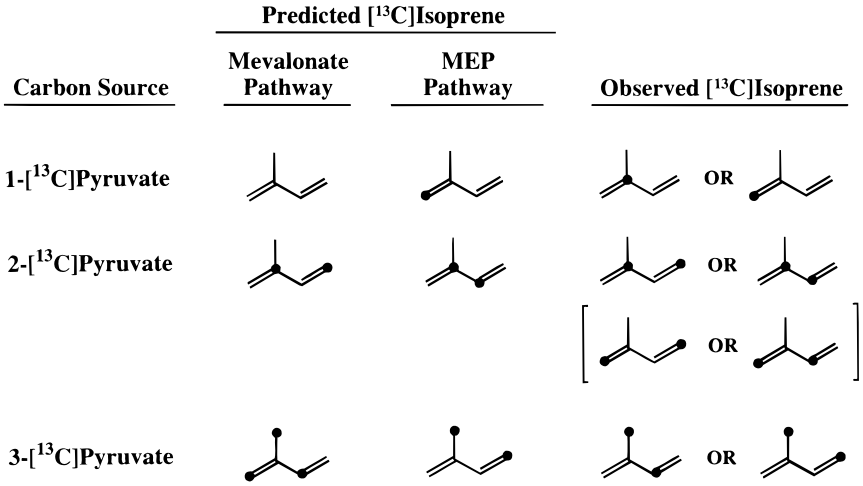

Figure 1. Predicted and observed ^{13}C-labeling of isoprene produced from *B. subtilis* 6051 grown on [^{13}C]pyruvate substrates. Predicted and observed ^{13}C-labeling patterns by both the mevalonate and MEP pathways are shown. Four possible observed [^{13}C]isoprene structures are shown for 2-[^{13}C]-pyruvate as carbon source; neither of the two structures in brackets is accounted for by these two pathways.

from amino acids, which were present in the medium; isoprene formation under growth conditions where glucose was absent are described below.

Parallel experiments with glucose selectively ^{13}C-labeled in the 1, 2, or 3 position were also conducted.[14] In these experiments ^{13}C incorporation in isoprene was lower due to the expected decreased number of labeled carbons and the dilution occurring at the triose phosphate step of glycolysis.[15] In addition, we observed partial labeling of the three major isoprene fragments, which prevented a clear distinction between the MEP and mevalonate pathways.[16] For these reasons these data are not presented here in favor of more definitive results obtained with the [^{13}C]-pyruvate labeling described below.

Isoprene labeling from U-[$^{13}C_6$]glucose cannot differentiate between the mevalonate and MEP pathways because both pathways originate from glycolytic intermediates (mevalonate from pyruvate via acetyl-CoA; MEP from pyruvate and glyceraldehyde 3-phosphate). In an attempt to achieve more specific isoprene ^{13}C labeling, we analyzed isoprene from *B. subtilis* 6051 cells grown on selectively labeled [^{13}C]pyruvates, including 1-, 2-, or 3-[^{13}C]pyruvate; the data obtained are summarized and compared to those obtained with unlabeled pyruvate in Table 1. To predict the labeling pattern of isoprene through the MEP pathway using [^{13}C]pyruvate, the regeneration of glyceraldehyde 3-phosphate through gluconeogenesis had to be taken into account. This was accomplished by tracing carbon atoms from pyruvate to oxaloacetate to phosphoenolpyruvate and back to glyceraldehyde 3-phosphate. Predicted labeling patterns for isoprene via the mevalonate pathway were straightforward, as acetyl-CoA is derived directly from pyruvate through the pyruvate dehydrogenase reaction.[17]

The detected parent and fragment ions (Table 1) allowed us to determine which isoprene carbons were labeled (Figure 1). As an example, when *B. subtilis* 6051 was grown on 3-[^{13}C]pyruvate, the isoprene produced exhibited major ion signals with masses of 69, 54, and 40, compared to unlabeled isoprene, which produced major signals of 67, 53, and 39 (Table 1). Because ion 69 became the major ion signal, two out of the five isoprene carbons were ^{13}C-labeled. One of these ^{13}C labels was contained in the $C_3H_3^+$ fragment 1, for ion 40 became the major ion signal rather than ion 39. The other ^{13}C label was contained in the $C_4H_5^+$ fragment, since ion 54 replaced ion 53 as the major signal. From the known isoprene fragmentation pattern, it follows that carbon 5 (methyl group) was labeled, and either carbon 3 or carbon 4 was labeled. This results in two possible ^{13}C-labeling patterns, one of which matches the [^{13}C]isoprene molecule predicted by the MEP pathway, as shown in Figure 1.

A similar analysis was performed on the [^{13}C]isoprene produced from *B. subtilis* 6051 grown on 1- or 2-[^{13}C]-pyruvate (Figure 1). Due to the fragmentation pattern of isoprene, a ^{13}C of either carbon 1 or carbon 2 cannot be resolved, nor can a ^{13}C label of either carbon 3 or carbon 4. These ambiguities lead to the possibility of the observed [^{13}C]isoprene compounds exhibiting one of two possible labeling patterns. Therefore, the ^{13}C-labeled isoprene obtained from 2-[^{13}C]pyruvate could have been derived from the mevalonate pathway. However, the results obtained with [^{13}C]isoprene produced from 1-[^{13}C]pyruvate and 3-[^{13}C]pyruvate do not support the patterns of isoprene labeling predicted by the mevalonate pathway, but are consistent with the MEP pathway.

As described earlier, the 2-methylbutane carbon skeleton of isoprene might be formed from a nonisoprenoid precursor. Because another *Bacillus* volatile, isoamyl alcohol (3-methyl-1-butanol), is likely to be formed by a leucine metabolic pathway,[18] we wanted to determine if leucine might be a precursor of isoprene. In these experiments we used deuteriomethyl-labeled leucines, including 5,5,5-[$^2H_3$]-leucine and isopropyl-[$^2H_7$]leucine, to determine if incorporation of deuterium into isoprene could be detected. Cells were grown under conditions to promote amino acid uptake and catabolism (i.e., tryptone was the sole carbon source) and supplemented with labeled leucine. Although the use of tryptone led to some dilution of the ^{2}H label, this was not a major problem as [^{2}H]leucines were diluted only by 9−16% from unlabeled leucine in the added Bacto−tryptone.[19] As shown in Table 1, we detected no deuterium incorporation into any of the three isoprene fragments produced by cells grown in the presence of 15 mM 5,5,5-[$^2H_3$]leucine or 27 mM isopropyl-[$^2H_7$]leucine. Even accounting for some loss of deuterium during leucine catabolism, these results indicate that under these growth conditions little or no isoprene was derived from leucine catabolism.

A key enzyme of the mevalonate pathway is hydroxymethylglutaryl−CoA reductase (HMGR), which catalyzes the formation of mevalonate.[10] We tested simvastatin, a specific inhibitor of the mevalonate pathway, for its ability to prevent *B. subtilis* growth. Simvastatin is a highly potent competitive inhibitor of HMGR;[20] it is an inactive pro-drug

containing a lactone ring, which, in humans, is converted to an active dihydroxy-open acid form in the liver. Simvastatin, in its acid form, has a K_i for HMGR of 0.5 to 1.0 nM. These types of HMGR inhibitors are also known to block the growth of archaea, which use the mevalonate pathway for isoprenoid biosynthesis.[21]

The lactone form of simvastatin was converted to the acid form by saponification and added in varying amounts to cultures of *B. subtilis* 6051. The minimum inhibitory concentration (MIC) required to inhibit cellular growth was 300 mM. Growth could not be rescued by adding mevalonate even at concentrations of up to 10 mM, an amount that did not inhibit cellular growth in the absence of simvastatin. Because the MIC of the acid form of simvastatin was so high, it is likely that the cells either lack the mevalonate pathway or the drug did not effectively enter the cells. The weak inhibition seen was probably a result of the drug affecting some other aspect of metabolism, because the addition of mevalonate could not rescue growth. We also tested the inhibitory effect of the lactone form of simvastatin and found that concentrations of up to 500 mM did not inhibit cellular growth. In contrast, growth of the archaeon, *Halobacterium salinarium* was completely inhibited by 1−10 μM simvastatin lactone.

As a second approach, we searched for the presence of the gene for HMGR in the *B. subtilis* genome.[9] Genes for this enzyme have been cloned from a variety of organisms, all of which share a high degree of homology. We performed homology searches of the *B. subtilis* genome[22] using human, yeast (*Saccharomyces cerevisiae*), bacterial (*Pseudomonas mevalonii*), and archaeal (*Sulfolobus solfataricus*) HMGR gene sequences.[23] None of these searches revealed any *B. subtilis* genes with significant homology to the HMGR sequences. This implies that *B. subtilis* does not contain an HMGR gene nor utilize the mevalonate pathway for isoprenoid biosynthesis. In contrast, the compound 1-deoxy-D-xylulose-5-phosphate is the first unique product of the pathways that lead to isoprenoids, thiamine, and pyridoxol synthesis;[24] reduction and isomerization of 1-deoxy-D-xylulose-5-phosphate to 2-*C*-methyl-D-erythritol 4-phosphate is thought to be the first unique step leading to IPP synthesis.[12] The *Escherichia coli* genes for 1-deoxy-D-xylulose-5-phosphate synthase and 1-deoxy-D-xylulose-5-phosphate reductoisomerase have been cloned, and in each case searches of the *B. subtilis* genome for these gene sequences have revealed the homologous genes.[12,24]

The goal of this work was to determine the pathway of isoprene biosynthesis utilized by *B. subtilis*, and four lines of evidence were obtained. First, [^{13}C]isoprene labeling from [^{13}C]pyruvates support the labeling patterns predicted by the MEP, but not the mevalonate pathway. The most revealing isoprene mass spectra results were derived from [^{13}C]isoprene produced from 1- and 3-[^{13}C]pyruvate. For these substrates even the ambiguities in ^{13}C-labeled isoprene fragments could not account for the formation of isoprene through the mevalonate pathway. Second, a concentration of simvastatin that inhibits HMGR in animals, yeast, and archaea does not inhibit *B. subtilis* growth. Third, the *B. subtilis* genome does not appear to contain an HMGR gene sequence, which would be essential if the mevalonate pathway were present. Fourth, the possibility that leucine degradation could be the source of the isoprene carbon skeleton was considered, but there was no detectable incorporation of deuterium from either 5,5,5-[$^2H_3$]-leucine or isopropyl-[$^2H_7$]leucine into isoprene under conditions where isoprene was formed during amino-acid degradation. These results support the model that *B. subtilis* utilizes the MEP pathway for isoprene biosynthesis. Additional support for this view comes from genomic evidence suggesting the presence of key genes for MEP pathway enzymes in *B. subtilis*.[12,24]

The absence of the mevalonate pathway in *B. subtilis* is somewhat surprising, given that intermediates of the mevalonate pathway have been detected in *Lactobacillus* and *Staphylococcus;*[25] these Gram-positive bacteria are usually regarded as taxonomically similar to *Bacillus* in the low GC subdivision of Gram-positive bacteria.[26,27] It seems likely that such closely related bacteria would use a common pathway for isoprenoid biosynthesis. However, it has been reported that the Gram-positive bacterium *Streptomyces aeriovifer* produces IPP by simultaneous operation of both mevalonate and nonmevalonate pathways.[28] In *S. aeriovifer* formation of the isoprenoid side chain of menaquinone appears to occur early in growth by the MEP pathway, and then an isoprenoid metabolite, naphterpin, is formed later in growth in a process sensitive to inhibition by HMGR inhibitors. Further investigation may reveal whether *Lactobacillus* and *Staphylococcus* also contain both MEP and mevalonate pathways, and the taxonomic significance of the presence of these different isoprenoid pathways in closely related low GC, Gram-positive bacteria.

The labeling results presented here are consistent with a C_5-prenyl diphosphate, such as IPP or DMAPP, as the immediate precursor to *B. subtilis* isoprene. This is analogous to the pathway for isoprene formation in plants, which possess the MEP pathway[11] and use DMAPP as a substrate for the enzyme isoprene synthase in chloroplasts.[2,29] Isolation and characterization of a *B. subtilis* isoprene synthase is needed to determine whether this bacterium converts DMAPP to isoprene, or whether it uses another C_5-prenyl diphosphate, such as IPP or 2-methyl-3-butenyl 2-diphosphate, the tertiary diphosphate analogue of DMAPP,[30] as the substrate. Because of its ease of genetic and physiological manipulation, further experiments on the biochemistry of isoprene formation in *B. subtilis* may shed light on the metabolic rationale for isoprene synthesis in higher organisms.

Experimental Section

General Experimental Procedures. [^{13}C]Glucose and sodium-[^{13}C]pyruvate compounds were obtained from Cambridge Isotope Laboratories, Inc. (Andover, MA). [5,5,5-[$^2H_3$]-Leucine and isopropyl-[$^2H_7$]leucine were obtained from C/D/N Isotopes Inc. (Pointe-Claire, Quebec, Canada). For analysis of isoprene labeling, 4-mL overnight precultures of *B. subtilis* 6051 were grown in F salts medium[8] containing 1% (w/v) [^{13}C]-labeled carbon source and 0.5% (w/v) Bacto−tryptone. Precultures were diluted to an OD_{600} value of approximately 0.1 into fresh medium (4 mL) containing ^{13}C-labeled substrate and grown at 37 °C on a rotary shaker until reaching an OD_{600} value between 0.5 and 0.8. Cultures were then transferred to 16-mL sealed vials and incubated for at least 2 h at 37 °C with shaking. Control experiments employed either unlabeled glucose, sodium pyruvate or L-leucine in place of ^{13}C- or ^{2}H-labeled substrates. A 10-mL sample of the headspace of each vial was drawn into a gas-tight syringe and analyzed by GC−MS with a modified configuration of the custom-made gas preconcentration system described in detail by Helmig et al.[31] The gas sample was injected through a septum inlet into a He gas flow and purged onto a cryogenic freeze-out trap consisting of a 0.53 mm i.d. × 30 cm length, uncoated, and deactivated fused silica column kept at −175 °C. Prior to the freeze-out of the sample, approximately 20 ng of a deuterated benzene internal standard were concentrated onto the freeze-out trap by purging a gas sample loop filled with the gas-phase

standard. The co-concentrated standard and collected volatiles were subsequently desorbed by rapid heating of the freeze-out trap to 75 °C and back-flushing onto a DB-1 fused silica capillary column (0.32 mm × 60 m, 1 μm film thickness, J&W Scientific, Folsom, CA). The GC (Hewlett−Packard HP 5890) oven temperature was kept at 0 °C during injection, held for 3 min, then ramped to 72 °C at a rate of 6 °C min^{-1} and then to 250 °C at a rate of 30 °C min^{-1}. Under these conditions isoprene eluted at approximately 8 min. Detection was accomplished with a Hewlett−Packard HP 5970 mass selective detector (MSD) operated in the electron impact (EI), 70 eV ionization mode, with a scan range from *m*/*z* 33 to 250. For determination of the minimum inhibitory concentration (MIC) of simvastatin, the drug was obtained as a gift from Merck and Co., Inc. Mevalonic acid lactone was obtained from Sigma Chemical Co (St. Louis, MO). Solutions of the sodium salts of simvastatin (dihydroxy-open acid form) and mevalonic acid were prepared by dissolving the lactone compounds in 100 μL of 95% (v/v) ethanol and adding 150 μmL of 0.1 N NaOH. The solution was heated at 50 °C for 2 h, followed by neutralization to a pH of 7.2 with 1 M HCl. This resulting solution was brought to a final volume of 1 mL. A solution of the lactone form of simvastatin was prepared in 75% (v/v) ethanol, or in 75% *N*,*N*-dimethylformamide for tests of inhibition of *B. subtilis* growth or isoprene formation, respectively, and diluted with water for use in each experiment. Precultures of *B. subtilis* 6051 were grown overnight in AB3 medium (17.5 g Antibiotic Medium 3 per liter). Overnight culture (50 μL) was added to 2 mL of fresh medium containing various concentrations of simvastatin and grown overnight at 37 °C on a rotary shaker. Bacterial growth was analyzed visually. The ability of mevalonate to rescue growth was tested by adding mevalonate to 2-mL cultures containing an inhibitory concentration of simvastatin. Inhibition of growth of *Halobacterium salinarum* 15900 by simvastatin was carried out as described elsewhere,[21] using growth medium 213 (American Type Culture Collection, Rockville, MD). Both bacterial strains were obtained from the American Type Culture Collection.

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