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Engineered Bacterial Production of Volatile Methyl Salicylate

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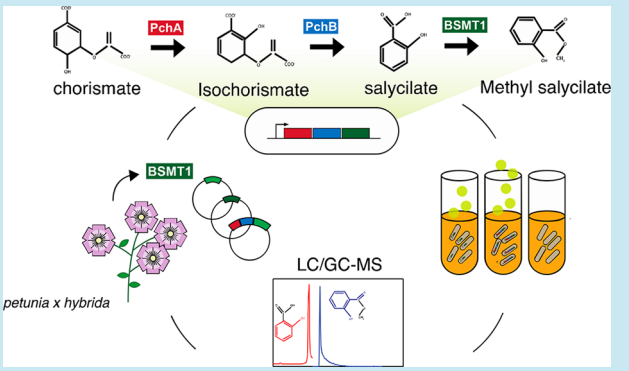
ABSTRACT: The engineering of microbial metabolic pathways over the last two decades has led to numerous examples of cell factories used for the production of small molecules. These molecules have an array of utility in commercial industries and as *in situ* expressed biomarkers or therapeutics in microbial applications. While most efforts have focused on the production of molecules in the liquid phase, there has been increasing interest in harnessing microbes' inherent ability to generate volatile compounds. Here, we optimized and characterized the production of methyl salicylate, an aromatic compound found mainly in plants, using a common lab strain of *E. coli*. We utilized genetic components from both microbes and plants to construct the volatile metabolite circuit cassette. In order to maximize production, we explored expression of methyl salicylate precursors, upregulation of expression by increasing ribosomal binding strength and codon optimization of the methyl transferase gene obtained from plant *Petunia x hybrida*. Last, we validated and quantified the production of methyl salicylate with liquid chromatography or gas chromatography mass spectrometry (LC-MS or GC-MS) and found that the codon optimized strain with precursor supplementation yielded the highest production compared to the other strains. This work characterizes an optimized metabolite producing genetic circuit and sets the stage for creation of an engineered bacteria diagnostic to be used in volatile assays.



KEYWORDS: *synthetic biology, metabolic engineering, methyl salicylate, chromatography*

Bacteria naturally produce volatile secondary metabolites that interact with other species and their surrounding environment during stages of biofilm formation, stress resistance, or virulence promotion.[1−4] With advances in metabolic engineering and synthetic biology techniques, microbes have been engineered as cellular factories for biomolecule production that give rise to an array of synthetic products ranging from artificial flavoring to therapeutics.[5−11] Due to bacteria's ability to specifically colonize certain regions of the body, such as the gastrointestinal tract and tumors,[12−16] they possess the capabilities for local production of diagnostic molecules with minimal systemic exposure. Indeed, natural bacterial volatile compounds have been used commonly in clinical settings for detecting dysbiotic or malignant disease.[17−23] Given their ability to synthesize volatile compounds and the existing infrastructure for detection of bacteria volatiles, we sought to engineer bacteria to produce methyl salicylate, for subsequent use as a diagnostic biomarker or other applications.

Methyl salicylate, commonly known as wintergreen oil, is naturally produced by plants upon stress induction.[24,25] It is a nontoxic and widely used molecule in a variety of food industries, is well-tolerated via inhalation, and can be detected sensitively via gas chromatography coupled to mass spectrometry. Additionally, no precursors of the methyl salicylate biosynthesis pathway are found in mammalian cells, which reduces the possibility of false positive signals in the context of a diagnostic molecule for human disease.[26] To engineer bacteria to synthesize the volatile methyl salicylate diagnostic marker, we utilized the *pchDCBA* operon and *BSMT1* gene in the genetic circuit. The *pchDCBA* operon is found in *Pseudomonas aeruginosa* genome and encodes for salicylate, an iron chelator of metal ions.[27,28] Specifically, *PchA* and *PchB* encode for isochorismate synthase and isochorismate pyruvate-lyase, respectively, which convert precursor chorismate to intermediate salicylate (Figure 1A). Chorismate is derived from fructose in the growth media, which can be metabolized by bacteria.[29] On the other hand, *BSMT1* is found in *Petunia x hybrida*, a flower plant.[30−32] The gene encodes for a methyltransferase that adds a methyl group to salicylate, forming the final product, methyl salicylate (Figure 1A).

We constructed two variants, pTC002 with the *BSMT1* gene and pTC003 with *PchA*, *PchB*, and *BSMT1* genes, both

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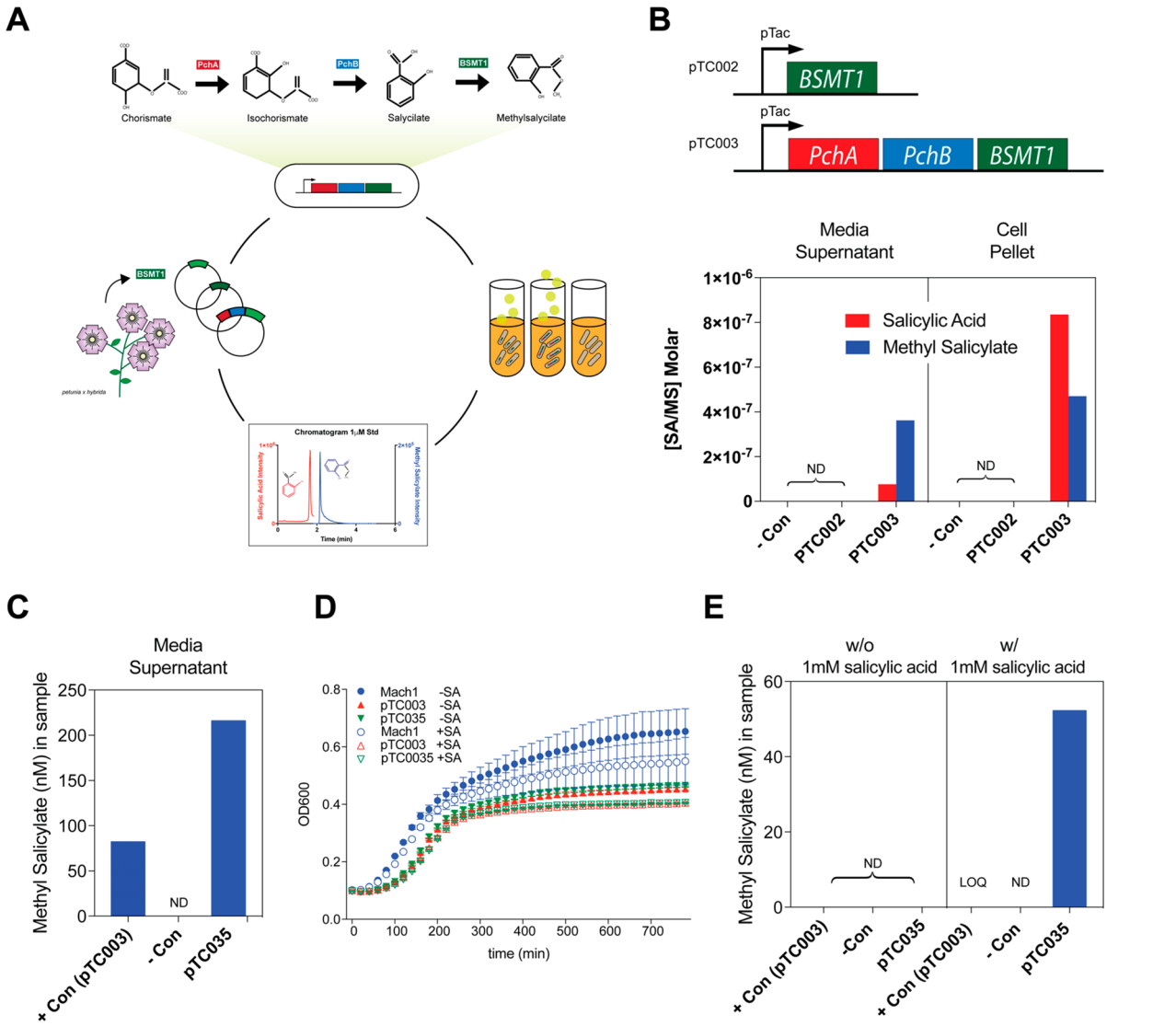

Figure 1. Characterization and quantification of methyl salicylate producing bacteria. (A) The methyl salicylate genetic circuit in *E. coli* utilizes the enzymes PchA and PchB to first convert precursor chorismate to intermediate salicylate. Specifically, isochorismate synthase, the product of the PchA gene, catalyzes the conversion of chorismate to isochorismate. The PchB gene, which expresses the isochorismate pyruvate-lyase, converts isochorismate to pyruvate and salicylate. The genetic circuit is completed with methyltransferase (BSMT1), which methylates salicylate to make the volatile ester methyl salicylate as the end product.[39] We then culture the bacteria strains and quantify volatile compound production by mass spectroscopy. Since, the BSMT1 gene is taken from the plant *Petunia x hybrid*, we codon-optimized it for our second iteration of optimization and repeated our quantification method. (B) Circuit design of pTC002 and pTC003 (top), overnight supernatant and cell pellet of bacteria strains along with negative control, *E. coli* strain with no engineered plasmid, collected and measured using LC-MS. Interpolated concentrations of SA or MS in experimental samples according to calibration curves. Concentrations represent the calculated levels of SA and MS in the media supernatant and cell pellet as labeled. ND = not detected. (C) Using GC-MS, pTC035 produced 2-fold more than pTC003. (D) Growth curves of bacteria in LB supplemented with ±1 mM salicylic acid across different strains (n = 3, mean ± SEM). (E) Measurement of methyl salicylate concentration from bacteria supernatant. Under stricter regiment, only pTC035 with 1 mM salicylic acid precursor added produced detectable amount. The limit of quantification (LOQ) is defined as three times the average of the analytical blanks plus a constant of 10 000 (arbitrary MS intensity units).

regulated by the P_{Tac} constitutive promoter (Figure 1B). We built these systems to explore whether a specific step in the biosynthetic pathway is the limiting reagent or critical factor to maximize methyl salicylate production. These plasmids were subsequently transformed in *E. coli Mach1* strain, a common lab strain that is widely used and easy to culture with rapid growth. Prior to sample characterization, we obtained standard curves of both salicylate and methyl salicylate (Supplemental Figure 1). Using liquid chromatography mass spectrometry (LC-MS), we were able to distinguish between salicylate and methyl salicylate (Supplemental Figure 2), which was not feasible by using ferric chloride colorimetric assay due to the overlapping chemical structure of the two molecules.[33] To characterize methyl salicylate production, we collected the supernatant and cell pellet of the different strains to perform mass spectrometry. As expected pTC003 produced an increased amount of both salicylate and methyl salicylate compared to pTC002, yielding methyl salicylate concentrations around 400 nM (Figure 1B). Interestingly, we observed more than 4-fold difference of salicylic acid in the cell pellet compared to the supernatant. We hypothesized that since salicylic acid is not volatile, it does not transport outside

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of the bacterial cell wall, therefore explaining the accumulation of salicylic acid found in the cell pellet.[25]

We verified bacterial production of methyl salicylate by performing LC-MS on the collected bacterial supernatant and cell pellets. In order to increase the production, we tuned the ribosomal binding site (RBS) strength to amplify transcription of the methyl salicylate cassette. In addition, since the methyl transferase gene (*BSMT1*) is taken from plants and preferential usage of particular codons varies by organism, we codon optimized the gene for bacteria in order to improve the translation efficiency of the gene; this new construct is named pTC035. Lastly, since methyl salicylate is a volatile compound, we measured the collected supernatant using gas chromatography mass spectrometry (GC-MS). By utilizing GC-MS and focusing on methyl salicylate in the gaseous state, we gained ∼500× sensitivity; therefore we did not quantify the nonvolatile salicylate. As predicted, the optimized genetic circuit pTC035 produced roughly 2-fold more than the non-codon-optimized version, pTC003, in cell supernatant (Figure 1C). Our results confirm that the pTC035 bacterial strain produces more methyl salicylate than nonoptimized construct pTC003 (Figure 1C), and we again validated, using GS-MS, bacterial production of methyl salicylate (Supplementary Figure 3).

We next explored adding salicylic acid in the growing bacterial culture as precursor in order to eliminate the possible rate limiting factor and show a proof of concept for boosting production in future *in vivo* work. We added 1 mM salicylic acid into bacterial cultures and first investigated whether it affected the growth rate or the viability of the cells (Figure 1D). After 12 h culturing, we noticed no difference between growth and concluded that 1 mM salicylic acid did not impede cellular growth. We then grew the strains overnight and collected supernatant for quantification. These extracts were analyzed by GC-MS and quantified by standard curve interpolation (Supplemental Figure 4). We found superior production from cultures with salicylic acid supplement compared with the same strain without, verifying our hypothesis that addition of precursor will increase our engineered metabolite production. Media supernatant from positive control pTC003 with salicylic acid was also detected but below the quantification threshold (Figure 1E).

Our proof of concept shown here demonstrates engineered bacterial production of methyl salicylate *in vitro*. We were able to verify methyl salicylate production using both LC-MS and GC-MS. In addition to characterization, we increased production of the gaseous compound by utilizing a stronger RBS, codon optimizing the plant derived methyl transferase gene, and supplementing with the pathway precursor, salicylic acid. Salicylate, hydrolyzed from salicylic acid, is an ingredient in common off the shelf pain killer aspirin and in cosmetic products, and is therefore regulated, safe, and well characterized as a precursor.[34−37] Further optimization of the production yield may include computational or experimental metabolic analysis to identify limiting factors of the pathway or enhancing enzyme function via directed evolution.[38] In the future, this system can be used as bacterially produced, volatile biomarker for translational diagnostics or other applications.

METHODS

Host Strain and Culturing. *E. coli Mach 1* strain was purchased from Thermos Fisher Scientific. All bacteria were cultured in LB media (Lennox) with respective antibiotic selection (100 μg mL^{-1} ampicillin) in a shaking incubator operating at 200 rpm and 37 °C for 12−16 h overnight until OD_{600} of 2−2.8. All bacterial cultures were OD_{600} matched to 2.0, followed by spinning down at 3000 rcf for 5 min in a centrifuge to collect the cell pellet and media supernatant. Samples were then stored at −80 °C prior to LC/GC-MS.

Plasmid Construction. *PchB*, *PchA*, and *BSMT1* were obtained by PCR from template plasmid iGEM part BBa_J45700. Codon optimized *BSMT1* was generated by the Codon Optimization Tool by IDT and also synthesized through IDT. All plasmids were constructed using Gibson assembly.

Metabolite Extraction. Methyl salicylate and salicylic acid were extracted from media samples and cell pellets in a 4 °C cold room as follows. For media samples, 200 μL of sample was diluted with 800 μL of acetonitrile to precipitate proteins and extract methyl salicylate. For cell pellets, the sample representing the cells from 1 mL of culture was extracted in 1 mL of 80% acetonitrile with mechanical bead lysis. Specifically, samples were collected in 2.0 mL screw cap vials containing ∼100 μL of disruption beads (Research Products International, Mount Prospect, IL). Each was homogenized for 10 cycles on a bead blaster homogenizer (Benchmark Scientific, Edison, NJ). Cycling consisted of 30 s homogenization time at 6 m/s followed by a 30 s pause. For both media and cell pellets, the resulting extracts were then centrifuged at 21 000*g* for 3 min, and the resulting supernatant was transferred to glass inserts for either LC-MS/MS or GC-MS analysis.

Liquid Chromatography−Tandem Mass Spectrometry. Salicylic acid and methyl salicylate were detected by a two-segment polarity switching targeted LCMS method. The LC column was a Waters BEH C18 (1.0 mm× 50 mm, 1.7 μm) coupled to a Dionex Ultimate 3000 system, and the column oven temperature was set to 25 °C throughout the 5 min isocratic (50%B) method. A flow rate of 100 μL/min was used with the following buffers: (A) 0.1% formic acid in water; (B) 0.1% formic acid in acetonitrile. Injection volume was set to 1 μL for all analyses. The LC output was coupled to a Thermo Q Exactive HF mass spectrometer operating in heated electrospray ionization mode (HESI). Data duration was 5 min with two PRM segments: negative mode from 0 to 1.8 min for salicylic acid and positive mode from 1.8 to 5 min for methyl salicylate. Spray voltage for both positive and negative modes was 3.5 kV, and capillary temperature was set to 320 °C with a sheath gas rate of 35, aux gas of 10, and max spray current of 100 μA. Each targeted PRM utilized 15 000 resolution for high frequency sampling with an automatic gain control (AGC) target of 3×10^6 and a maximum IT of 500 ms, fixed first mass of 50 m/z, isolation window of 0.4 m/z, and fixed HCD energy (nCE) of 35. All data were acquired in profile mode, and peak heights were detected with a custom Thermo Quan browser method developed with authentic standards.

Gas Chromatography−Mass Spectrometry. Methyl salicylate was detected using a split injection method on a Thermo TRACE 1310 gas chromatograph with a split ratio of 25 and an injection volume of 1 μL where the split flow was 25.0 mL/min for all samples and standards. A constant temperature of 250 °C was used for the injector in combination with a Topaz low pressure drop precision liner with wool (Restek). The carrier gas was helium at a constant flow of 1.0 mL/min with a Thermo TG-WAXMS A column (30 m × 0.25 mm). The thermal gradient profile was as follows: equilibration of 2 min followed by a 1 min hold at 100

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°C, then a ramp from 100 to 145 °C at 20 °C/min followed by a 4 min hold at 145 °C, then a ramp from 145 to 165 °C at 15 °C/min followed by a 3 min hold at 165 °C. Analyses were carried out by coupling the GC system to a Thermo Q Exactive mass spectrometer operating in electron ionization (EI) positive mode at 70 keV. Method duration was 15 min. An MS1 scan range from 32 to 350 m/z was used at a resolution of 120 000 with an AGC target of 1×10^6 and a maximum IT of 100 ms. All data were acquired in profile mode and tracked the 120.0206 m/z (theoretical) characteristic EI fragment of methyl salicylate representing the $[C_7H_4O_2]^+$ moiety (loss of methoxy group). The parent ion at 152.0458 m/z (theoretical) was also tracked as a confirmation ion $[C_8H_8O_3]^+$.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssynbio.0c00497.

LC-MS/MS quantification of standards, chemical structure identification of compounds via MS, methyl salicylate GC-MS confirmation and standard curves by GC-MS (PDF)

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Author Contributions

T.C. and T.D. conceived and designed the study. T.C. and D.R.J. performed *in vitro* characterization of methyl salicylate circuit. T.C., D.R.J., and T.D. wrote the manuscript.

Notes

The authors declare no competing financial interest.

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