E. coli Metabolic Engineering for Gram Scale Production of a Plant-Based Anti-Inflammatory Agent

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Abstract

In this report, the heterologous production of salicylate (SA) is the basis for metabolic extension to salicylate 2-*O*-*β*-D-glucoside (SAG), a natural product implicated in plant-based defense mechanisms. Production was optimized through a combination of metabolic engineering, gene expression variation, and co-culture design. When combined, SA and SAG production titers reached ~0.9 g/L and ~2.5 g/L, respectively. The SAG compound was then tested for anti-inflammatory properties relative to SA and acetylsalicylate (aspirin). Results indicate comparable activity between SAG and aspirin in reducing nitric oxide (NO) and reactive oxygen

species (ROS) from macrophage cells while no discernable negative effects on cellular viability were observed.

Keywords: Salicylate, Salicylate 2-*O*-*β*-D-glucoside, Plant, *E. coli*, Anti-inflammatory, Co-culture

1. Introduction

Natural products can be divided into classifications characterized by complex molecular architectures that require similarly complex biosynthetic pathways(Weissman and Leadlay, 2005). A prime example is the modular enzymatic machinery responsible for polyketide and nonribosomal peptide compounds(Cane, 1999; Fischbach and Walsh, 2006; Katz, 1997). The primary motivation in producing such products stems from their medicinal value which spans antibiotic, anticancer, and immunosuppressive activities(Demain, 2009; Demain, 2014; Demain and Sanchez, 2009).

A technical challenge in the pursuit of such compounds is the fastidious nature of many native production hosts(Zhang et al., 2011b). As a result, limits are placed on both initial product access and opportunities for molecular engineering afforded by the unique biosynthetic mechanisms responsible for product formation. Issues include poor or impossible host culturability, genetic intractability, slow growth kinetics or lack of cellular density, complex cellular morphology, rudimentary or non-existent molecular biology protocols, and native pathogenicity. Natural product value confounded by drawbacks of a native host prompted the approach of heterologous biosynthesis in which the genetic material of a target natural product is transferred to a surrogate host capable of allowing compound access and greater engineering opportunity(Ongley et al., 2013).

This same concept can be applied to less complex compounds that hold value as the basis for commodity or pharmaceutical chemical production, driving the concept of a bio-based economy(Yadav and Stephanopoulos, 2014; Zhuang and Herrgard, 2015). As an example relevant to the current work, salicylic acid is the precursor to the ubiquitously used acetylsalicylic acid (i.e., aspirin), a non-opioid and non-steroidal anti-inflammatory medicine with an estimated 40,000 metric tons produced yearly(Warner and Mitchell, 2002). However, the predominant source of salicylic acid driving commercial aspirin production is petroleum-based, which leads to questions of long-term sustainability(Lindsey, 1957).

In this case, biosynthetic production of SA would offer an alternative production route with the option to leverage the cellular background for additional metabolic and molecular engineering. In our previous work to heterologously produce the nonribosomal peptide-polyketide siderophore yersiniabactin, we introduced an *irp9* gene from *Yersinia enterocolitica* to enable the production of SA from *E. coli*(Kamal Ahmadi et al., 2015; Pelludat et al., 2003) (Figure 1). The SA support pathway provides the basis for the current work dedicated to engineering the heterologous production of a new compound, salicylate 2-*O*-*β*-D-glucoside (SAG) using genetic material transferred from a plant source. This background also highlights the utility of *E. coli* heterologous biosynthesis in addressing native host limitations which include pathogenicity (in the case of yersiniabactin production) and culturability/growth kinetics/genetic tractability (in the case of SAG).

More specifically, we introduced a gene from *Arabidopsis thaliana* to convert heterologous SA to SAG. The final pathway was then optimized through the application of metabolic engineering, gene expression design, and co-culturing techniques to enable multi-gram per liter production levels of the final compound. In vitro assays confirmed preliminary anti-

inflammatory properties when compared to aspirin and support a bio-based platform for the potential mass production of this compound.

2. Materials and methods

2.1 Medium components and molecular biology reagents

All culture media, sample preparation, and analytical components were purchased from Fisher Chemical (Pittsburgh, PA, USA) or Sigma-Aldrich (St. Louis, MO, USA) unless otherwise indicated. DNA-manipulating agents, including restriction enzymes, T4 DNA ligase, Phusion High-Fidelity PCR Master Mix, and associated reagents were obtained from New England Biolabs (Ipswich, MA, USA). PCR primers were obtained from Eurofins Genomics (Huntsville, AL).

2.2 Strains and plasmids

Strains, plasmids, and PCR primers used in this study are presented in Tables S1 and S2. Cloning procedures were completed using *E. coli* DH5α. The *irp9* gene was amplified from *Y. enterocolitica* genomic DNA, and the *pgm* and *galU* genes were amplified from the *E. coli* K-12 MG1655 genome. The amplified genes were digested with restriction enzymes *Nde*I/*Hin*dIII (for *irp9* and *pgm*) and *Nhe*I/*Hin*dIII (for *galU*) and separately ligated into similarly digested pET28a to yield pET28-*irp9*, pET28-*pgm*, and pET28-*galU*. Plasmids pET28-*irp9*-1361 and pET28-*irp9*-3231 were constructed with the same cloning strategy using semi-synthetic pET28a vectors containing altered ribosome binding sites(Espah Borujeni et al., 2014; Salis et al., 2009). Plasmid pET28-*pgm* was digested with *Xba*I/*Hin*dIII and transferred to *Spe*I/*Hin*dIII digested pET28-*galU* to generate pET-30. Plasmid pET-30 was digested with *Xba*I/*Hin*dIII and ligated to *Spe*I/*Hin*dIII digested pET28-*irp9*, pET28-*irp9*-1361 and pET28-*irp9*-3231 to construct pET-31, pET-1361, and pET-3231, respectively. The pET28-based constructs were then transferred to

the pBAD33 vector using the *Xba*I/*Hin*dIII restriction sites. The SA glucosyltransferase gene (*ugt74f1*) from *A. thaliana* (Gene ID: 818988) was codon optimized and synthesized by GenScript (Piscataway, NJ, USA) and cloned into the pGEX-2TK vector with the *Bam*HI and *Eco*RI restriction sites(Lee and Raskin, 1999; Lim et al., 2002; Song, 2006).

Single knockout strains were obtained from the Keio collection (Coli Genetic Stock Center, Yale University)(Baba et al., 2006). Double and triple knockouts were further constructed using standard P1 transduction followed by antibiotic (Kan^r) marker removal using the pCP20 plasmid(Datsenko and Wanner, 2000). Knockout strains were confirmed by PCR. Plasmids were transformed using electroporation and resulting strains were stored as 20% glycerol stocks at -80°C.

2.3 Culture conditions for heterologous production

Glycerol stocks were used to inoculate overnight cultures incubated at 37°C with shaking in lysogeny broth (LB) medium which were then used to inoculate (1% v/v) 25 mL of M9Y medium (per liter: 12.8 g Na_2HPO_4•7 H_2O; 6 g Na_2HPO_4; 3g KH_2PO_4; 0.5 g NaCl; 1 g NH_4Cl; 1 g yeast extract, 10 g glycerol, 2.5 g glucose, 246.5 mg $MgSO_4$•7 H_2O, 14.7 mg $CaCl_2$•2H_2O) supplemented with micronutrients including (per liter) vitamin B1 (2.0 mg), H_3BO_3 (1.25 mg), $NaMoO_4$•2 H_2O (0.15 mg), $CoCl_2$•6 H_2O (0.7 mg), $CuSO_4$•5 H_2O (0.25 mg), $MnCl_2$•4 H_2O (1.6 mg), and $ZnSO_4$•7 H_2O (0.3 mg). Post-inoculation, cultures were incubated at 30°C with shaking for 2 days with induction initiated at an OD_{600nm} of 0.4–0.6 using 200 µM isopropyl β-D-1-thiogalactopyranoside (IPTG) and 3 mg/mL arabinose, as needed. Culture plasmid selection was maintained with 100 mg/L ampicillin, 50 mg/L kanamycin, and 20 mg/L chloramphenicol, as needed (the same antibiotic levels were used for solid medium transformation selection).

2.4 Salicylate (SA) and salicylate 2-O-β-D-glucoside (SAG) production quantification

Post-culture, a 1 mL sample was centrifuged and 20 µL of supernatant analyzed by HPLC(Dean et al., 2005). SA and SAG were quantified using a ZORBAX Eclipse XDB-C18 column connected to an Agilent 1100 system equipped with a diode array detector. Solvent A was 0.1% acetic acid in water, solvent B was methanol, and a flow rate of 1 mL/minute was used across the following gradient: 5 to 50% solvent B over 20 minutes; 50 to 80% solvent B over 5 minutes; 80% solvent B maintained for 5 minutes; reset to 5% solvent B. Absorbance wavelengths of 304 and 274 nm were used for SA and SAG quantification, respectively. Peak area quantification was conducted compared to a standard calibration curve of pure SA (Sigma-Aldrich) and SAG (Toronto Research Chemicals, Toronto, ON, CAN). The same analytical method was used to measure SAG degradation over time by adding 400 mg/mL SAG to a culture of BW/pBAD33/pET21a incubated in M9Y medium at 30°C for 48 hr.

2.5 Biological interaction assays

Cell growth data measured by OD_{600nm} values across *E. coli* strains were collected within 96-well plates containing 200 µL of M9Y medium. Glycerol stocks were used to inoculate overnight cultures incubated at 37°C with shaking in LB medium which were then used to inoculate (1% v/v) individual wells for subsequent culture at 30°C for 2 days. SA and SAG were added at an OD_{600nm} value of 0.4. A Synergy 4 Multi-Mode Microplate Reader (BioTek Instruments, Inc.) was used to record values for all assays listed in this sub-section.

Cytotoxicity of SA, aspirin (ASP), SAG, and heterologously-produced SAG (B-SAG) was determined by the 3-(4, 5-dimethylthiazol-2-yl)-diphenyltetrazolium bromide (MTT) colorimetric assay. The RAW264.7 cells used in the assay were maintained in medium prepared as follows: 50 mL of FBS (heat inactivated), 5 mL of 100 mM MEM sodium pyruvate, 5 mL of 1 M HEPES buffer, 5 mL of penicillin/streptomycin solution, and 1.25 g of D-(+)-glucose added

to 500 mL RPMI-1640 and filter sterilized in T75 flasks at 37°C/5% CO_2. Cells were harvested using mechanical scrapers prior to seeding at 3×10^4 cells/well in tissue culture-treated, sterile, polystyrene 96-well plates in 100 μL medium per well. Varying concentrations of each compound were added and cells incubated for 24 h before viability analysis. For these and the assays described below, B-SAG was purified by collecting samples from the HPLC method described above.

Nitric oxide (NO) production from RAW264.7 cells was determined using a Griess reagent kit (Promega, Madison, WI, USA). Cells were cultured and collected as indicated above for seeding at 5×10^4 cells per well in a 96-well plate. SA, ASP, SAG, and B-SAG were then applied for 1 h, followed by the addition of LPS (1 μg/mL). After 18 h, NO production was measured.

To assess intracellular reactive oxygen species (ROS) production, the fluorescent probe 2′,7′ dichlorodihydrofluorescein diacetate (DCFH-DA) was used. RAW264.7 cells were seeded (5×10^4 cells per well) in 96-well plates and treated with different concentrations of SA, ASP, SAG, and B-SAG for 1 h before stimulation with LPS (1 μg/mL) for 18 h. DCFH-DA (10 μM) was then added and incubated for an additional 45 min before ROS production was measured through fluorescence output with an emission wavelength of 525 nm and excitation wavelength at 488 nm. ROS production was compared to untreated and LPS-stimulated RAW264.7 cells.

2.6 Statistical evaluation

Data presented were generated from three independent experiments, and error bars represent standard deviation values. Statistical significance comparisons between indicated groups were performed using a one-way ANOVA with Dunnett post-tests.

3. Results and discussion

3.1 Improved heterologous salicylate production through metabolic engineering

The Irp9 enzyme from *Y. enterocolitica* is capable of converting chorismate to isochorismate while also enabling subsequent conversion to SA (Figure 1)(Kerbarh et al., 2005). Upon introducing this enzyme to *E. coli* in the context of efforts to support production of the complex natural product yersiniabactin, un-optimized production of SA afforded by this step reached ~300 mg/L(Kamal Ahmadi et al., 2015).

The initial heterologous production levels of SA achieved through the introduction of *irp9* were considered a strong starting point for future efforts to improve titers. In this work, metabolic engineering was applied as a first means to do so. Specifically, the pathways dedicated to amino acid production from chorismate were eliminated through gene deletion (Figure 1). This was accomplished by testing previously constructed knockout mutants available through the Keio collection together with a plasmid 1) compatible with the selection marker used within the single knockout Keio strains and 2) expressing the *irp9* gene from an arabinose-inducible promoter. Using this system, the highest salicylate production levels were achieved with the *tyrA* mutant (Figure 1). Interestingly, in this analysis, initial production from the wildtype BW25113 (BW) strain was ~420 mg/L, substantially higher than the level obtained previously using a B strain of *E. coli* which may reflect the intrinsic differences between strains or the switch to a pBAD expression system.

Combination mutants were then assembled by eliminating the kanamycin resistance marker from target host knockout strains prior to P1 phage transduction from separate knockout strains. The approach allowed the systematic assessment of double and triple deletion mutants. In this analysis, the *pheA*/*tyrA* double deletion mutant (BW23) produced the highest SA titers, which supports results from the single deletion assessment by coupling the two mutants with the

highest individual SA titers and increasing production through double deletion. SA production levels reached ~900 mg/L using the *pheA*/*tyrA* double mutant. Interestingly, inclusion of the *trpD* mutant in all cases (single to triple deletion mutants) depressed final SA levels relative to the wildtype strain or the mutants that enhanced production. Thus, the *pheA*/*tyrA* double mutant was utilized in subsequent efforts to boost SA and SAG production.

Separate research to establish SA production in *E. coli* used a combination of native and heterologous metabolism to generate isochorismate and SA, and initial SA production levels reached 158.5 mg/L(Lin et al., 2014). A strength of the Irp9 approach utilized in the present study is the ability to address both steps with the added benefit of initial production levels of 300 mg/L SA (using a B strain) and ~420 mg/L (using a K strain). Interestingly, previous efforts to boost SA production through the triple *pheA*/*tyrA*/*trpD* mutant also saw reduced titers compared to efforts with the *pheA*/*tyrA* mutant(Lin et al., 2014). This same study noticed a reduction in cell density when using the triple mutant. We see a similar trend as indicated by final and time course assessment of cellular density levels (Figures 1 and S1). The same previous study used additional metabolic engineering of the shikimate pathway to obtain an SA titer of 1180 mg/L; however, greater titers were hampered by *E. coli* toxicity caused by SA which limited production to ~1,000 mg/L. Our upper limits of ~900 mg/L SA similarly support a limit to production through *E. coli* without efforts to develop increased tolerance.

3.2 Extension of heterologous biosynthesis for SAG production

Figure 2 summarizes initial efforts to establish SAG production from *E. coli*. Here, an SA glucosyltransferase from *A. thaliana* was synthesized and codon optimized for *E. coli* co-expression with *irp9*. In these efforts, two additional native *E. coli* genes, *pgm* (encoding phosphoglucomutase) and *galU* (encoding UDP-glucose pyrophosphorylase), were included to

improve pathway flux towards UDP-glucose(Mao et al., 2006). Initial production levels from the wildtype *E. coli* base strain resulted in ~1,250 mg/L SAG, which increased to ~1,500 mg/L upon the inclusion of *galU* and *pgm*. When the same expression constructs were tested in the *pheA*/*tyrA* double mutant identified from Figure 1, production levels of SAG improved to ~1,600 and ~2,100 mg/L without and with *galU*/*pgm*, respectively. Using the double mutant strain, conversion of SA to SAG is indicated via HPLC analysis (Figure 2B).

It is interesting that initial production levels of SAG topped those of the highest levels of SA achieved from the mutant analysis presented in Figure 1. We suspect this is due to the documented growth inhibition caused by SA production(Lin et al., 2014). In Figure S2, we observe the previously noted negative effect upon cell growth caused by SA and the lack of inhibition upon the addition of SAG. Hence, the metabolic outlet of SAG formation offers the opportunity for higher production levels as a result.

The final construct tested in SAG production (pMKA-41) was chosen from three options containing different RBS translation initiation rates (TIRs). In so doing, the goal was to alter SA production and enable complete conversion to SAG. Specifically, three different RBS sequences were compared, each providing a different TIR for *irp9*. As indicated in Figure 3, the construct containing the highest TIR, produced the most SAG and, hence, this plasmid was the basis for the additional studies described to further enhance production. Residual SA observed across reduced TIRs also suggests that the SAG conversion step is rate limiting.

3.3 Co-culture utilization in SAG production

Co-culture techniques have recently been used to great effectiveness with systems that may benefit from a biosynthetic pathway divided between two strains(Zhang et al., 2015; Zhang and Wang, 2016). Here, we applied this concept in an effort to more completely convert SA to

SAG since residual SA levels result from our initial attempts to consolidate the SAG pathway to one cell (Figure 2B and 3). In an initial attempt to apply the co-culture approach, the SAG pathway was divided between SA and SAG production (Figure 4). Though successful co-culture was demonstrated by a shift to SAG production upon strain ratio variation, final SAG titers leveled to ~900 mg/L, approximately half the value obtained during pathway single cell consolidation (Figure 2). Though it is unclear why similar production levels could not be achieved in a co-culture format, the issue may be an overabundance of SA having deleterious effects on cell growth prior to conversion to SAG or a dilutive effect on the initial SA producer as cellular ratios are varied.

As such, a new co-culture approach was adopted based upon the diagram provided in Figure 5. Here, the first cell would be that optimized for consolidated SAG production, noting that residual SA will also be produced. Thus, the addition of a second strain designed to only convert SA to SAG would then be expected to maximize final production. This was tested over a range of co-culture mixtures with the 20:1 ratio providing optimal production of SAG (~2,500 mg/L). Higher ratios between strains are likely diluting the effect of strong initial SAG production from the pathway consolidation strain. However, the new maximum level of SAG highlights the utility of co-culturing to extend production potential.

Future optimization efforts can build upon the work here by balancing the single cell production of SA and SAG to similarly maximize conversion. Options to do so include carefully balancing the expression of the genetic components required for SA and SAG. In so doing, final cellular constructs could then be coupled to process options that include fed-batch or continuous bioreactor configurations to further extend SAG production.

3.4 Bioactivity assessment of SAG

SAG formation has been implicated as part of innate plant-based defense mechanisms, with SAG postulated as a storage form for degradation and release of SA(Rivas-San Vicente and Plasencia, 2011; Vlot et al., 2009). Here, we observed only a slight degradation (~15%) of SAG over time within a production culture background (Figure S3). Close analogs of SAG have also shown potential as an anti-inflammatory agents(Xin et al., 2013; Zhang et al., 2011a). As such, the SAG heterologously produced from *E. coli* in this study was tested across two assays related to inflammation, with aspirin used as a key comparison point. The first assay was nitric oxide (NO) production from macrophage RAW264.7 cells. SAG performs better or comparable relative to aspirin across all concentrations tested with both SAG and aspirin showing statistically improved reduction in NO levels compared to SA (Figure 6A). Similarly, reduction in reactive oxygen species (ROS) from RAW264.7 cells is provided by SAG at levels comparable or better than aspirin while significantly better than SA (at the two highest concentrations tested; Figure 6B). These results indicate the early potential of SAG as a viable anti-inflammatory agent produced through a biological route.

As importantly, SAG must not show unwanted toxicity towards mammalian cells. This was preliminarily assessed through viability retained by the RAW264.7 cells upon compound exposure. In this regard, all three compounds tested exhibited minimal effect to cellular viability with SAG showing a slight, though insignificant, improvement relative to SA and aspirin at the highest concentration tested (Figure 6C). Viability maintained over the concentrations tested for anti-inflammatory effects further supports the future therapeutic potential of SAG.

4. Conclusions

Biosynthesis of a plant-based anti-inflammatory agent (SAG) was accomplished using *E. coli* as a heterologous host. Metabolic engineering was applied and translation initiation rates varied to optimize the production of SA and SAG. Co-culture methodology was then utilized to further promote the complete conversion of SA to SAG, allowing final production of SA to reach ~2.5 g/L. Bioactivity assessment of SAG confirmed anti-inflammatory properties of NO and ROS reduction from RAW264.7 macrophage cells at a potency comparable or better than aspirin while showing no preliminary adverse effects to cellular viability, thus, offering the potential to generate and utilize SAG as a bio-based alternative.

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Figure captions

Figure 1. Metabolic engineering to improve SA production. Strains containing individual and combined *tyrA*, *trpD*, and *pheA* mutants are assessed for SA production (bar graph). *statistical significance at 95% confidence level compared to production from strain BW/pBAD-*irp9*.

Figure 2. Heterologous SAG production. (A) The introduction of UGT74F1 and the GalU and Pgm metabolic steps (combined for gene expression from pMKA-41) both enable and improve production of SAG. (B) HPLC trace of SA and SAG heterologous production compared to authentic standards. *statistical significance at 95% confidence level when comparing corresponding samples across the BW and BW23 strains.

Figure 3. SAG production comparison across RBS translation initiator rates (TIRs). SAG levels across the indicated RBS sequences and TIRs associated with *irp9*. Statistical significance at 95% confidence level when comparing pMKA-3231 to pMKA-1361 (*) and pMKA-41 to pMKA-3231 (**).

Figure 4. Co-culture application to SAG production. Ratios of the indicated strains were varied in an effort to convert SA completely to SAG.

Figure 5. Co-culture conversion of residual SA. Residual SA from a consolidated production system was converted through a secondary strain dedicated only to SAG formation. *statistical significance at 95% confidence level when comparing SAG production from 20:1 to 20:0 ratios.

Figure 6. Anti-inflammatory and cellular viability assessment of SAG. Raw264.7 macrophage NO (A), ROS (B), and viability (C) measurements when treated with SA, aspirin (ASP), SAG, and B-SAG (produced heterologously). Statistical significance at 95% confidence level when comparing B-SAG to SA (*).