Undergraduate Journal of Experimental Microbiology and Immunology (UJEMI) Vol. 27:1-14


# Multiple deletions arise from attempted insertion of the salicylate *nahR/Psal* biosensor into a duo-directional SIGEX vector

Kaya Frese, Kitty Martens, Tamara Nichvolodoff

Department of Microbiology and Immunology, University of British Columbia, Vancouver, British Columbia, Canada

**SUMMARY** To address the ongoing problem of plastic accumulation in marine environments, there is a need to identify microbial communities capable of degrading plastic associated pollutants and characterize the inducible systems that they use for applications in synthetic biology. Substrate-induced gene expression reporters have been used as a screening tool to find inducible promoters sensitive to plastic-associated compounds. A duo-directional substrate-induced gene expression reporter (pSPPH21), with fluorescent reporter proteins on either side of the insertion site, is theoretically capable of searching for both uni- and bi-directional promoters and would function independent of the insert orientation. This study evaluates the functionality of pSPPH21 as a tool to screen environmental samples for promoters neighboring plastic-degrading enzymes. To this end, the *nahR/Psal* bi-directional promoter system was inserted into pSPPH21 (pSPPH21::*nahR/Psal*) and subsequently transformed into competent *Escherichia coli* cells. Colonies were induced with salicylate, a breakdown product of naphthalene, and measured for fluorescence activity. Colonies with the pSPPH21::*nahR/Psal* construct generally responded to salicylate induction in a concentration dependent manner. A response was not observed by salicylate alone or upon induction of the empty pSPPH21 vector. In practice, the *nahR/Psal* biosensor did not respond to water stored in a polyethylene terephthalate container for 6 days. Despite the observed responses, sequencing results of the tested colonies displayed no insertion of the *nahR/Psal* fragment at the NruI site and multiple deletions within or surrounding the NruI site.

## INTRODUCTION

Microbial biosensors - here defined as microbes that are capable of inducing gene expression in response to a particular chemical signal - have been proposed as a way to address the ongoing problem of plastic accumulation in marine environments (1). Environmental marine microbes are capable of degrading polyethylene (PE) and polyethylene terephthalate (PET) (2,3). Colonization of plastics by microbial communities occurs in as little as two weeks in the ocean environment, and biodegradation can occur in a variety of plastic types (4). Inducible gene expression of enzymes in response to the presence of a specific substrate in the environment increases the fitness of an organism by allowing it to elaborate degradative enzymes only when their specific substrates are present (5). Screening marine microbes for the capacity to act as biosensors for plastic-associated contaminants may therefore help to both identify microbial community members capable of degrading plastic compounds and to identify novel inducible systems that could be used in synthetic biology applications. The breakdown of PET produces similar compounds to salicylate, which can act as an inducer of gene expression in the *nahR/Psal* system (6,7). This system is a BioBrick registered in the iGEM Registry of Standard Biological Parts as BBa_J61051 (http://parts.igem.org/partsdb/get_part.cgi?part=BBa_J61051).

Uni-directional substrate-induced gene expression (SIGEX) has been applied to screen for promoters in environmental samples (8). In this system, a restriction site is placed upstream of a reporter gene with an associated ribosome binding site (RBS) in a plasmid, and random fragments of sample DNA are inserted at the restriction site. The plasmid is then introduced into model bacteria, such as *Escherichia coli*, and the bacteria are screened for expression of fluorescent protein in response to substrates of interest. Cells that express the

**Published Online:** September 2022

**Citation:** Kaya Frese, Kitty Martens, Tamara Nichvolodoff. 2022. Multiple deletions arise from attempted insertion of the salicylate *nahR/Psal* biosensor into a duo-directional SIGEX vector. UJEMI 27:1-14

**Editor:** Andy An and Gara Dexter, University of British Columbia



Address correspondence to: https://jemi.microbiology.ubc.ca/

reporter after substrate induction are likely to contain inducible promoters sensitive to that substrate. Those promoters are in turn likely to neighbor degradative enzymes for that substrate. Duo-directional SIGEX - containing fluorescent reporter proteins on either side of the insertion site - can identify inserts containing bi-directional promoters and function independent of the orientation of the inserted fragment (9,10). The duo-directional SIGEX reporter vector (pSPPH21) contains a NruI restriction enzyme recognition site flanked by green fluorescent protein (GFP) and red fluorescent protein (RFP) reporter genes in opposing orientations (9). Since NruI makes blunt ended cuts, there is no specific sequence requirement for the insertion fragments other than they are blunt ended (11). If both RFP and GFP are expressed, the result would be an amber-coloured signal, allowing for identification of overlapping promoters in opposing orientations or duo-directional promoters (12). Though this construct was created and partially sequenced, it has not been used to evaluate inserts for promoter activity.

It has been reported that salicylate was able to induce gene expression through the *Psal* promoter in a NahR-dependent manner, which in turn initiated transcription of enzymes involved in salicylate metabolism to TCA cycle intermediates (13). A pSPPH21::*nahR/Psal* construct transformed into *E. coli* DH5α or TOP10 *E. coli* cells are expected to yield isolates expressing either GFP or RFP under induction by salicylate, consistent with the orientation of the insert as determined by sequencing. This characterized inducible system will be used to determine if pSPPH21 is capable of identifying the orientation and activation state of an inducible promoter introduced at the NruI site.

## METHODS AND MATERIALS

### Bacterial strains and plasmids

The pSPPH21 plasmid was isolated from *E. coli* DH5α cells that were obtained from a frozen glycerol stock from a previous UJEMI student group (P. Abrishamkar, H. M. Dana, S. Oveisi, and P. Tabassi, submitted for publication). Subcloning Efficiency™ DH5α Competent Cells (Invitrogen) and One Shot™ TOP10 Chemically Competent *E. coli* (Invitrogen) cells were used for transformation. The positive controls used in the fluorescence assay were *E. coli* DH5α cells that contained the pSPPH21 plasmid with the *lac* promoter, along with *lacI*, inserted in either the direction of GFP or RFP at the NruI site and were obtained from Gawol *et al.* from a parallel pSPPH21 project (D. Gawol, R. Floyd, K. Kohara, and Y. Lee, submitted for publication). All cells were grown at 37°C on Luria Bertani (LB) agar plates or in LB liquid media both containing 25 μg/mL chloramphenicol. The recipes for LB broth and chloramphenicol stock were adapted from Green & Sambrook (14).

### PCR amplification of *nahR/Psal*

The *nahR/Psal* gene fragment (no 5' phosphorylation) was ordered from Integrated DNA Technologies (IDT) and reconstituted in sterile dd$H_2O$. The *nahR/Psal* sequence can be found at https://github.com/TwirlingTowardsFreedom/MICB-401---Team-1Theta---Sequencing-Results-DNA-files/. Polymerase Chain Reaction (PCR) was performed using Platinum™ SuperFi™ DNA Polymerase according to the manufacturer's instructions (Invitrogen). The *nahR/Psal* forward (5' GGCCGCTGCGATCCC 3') and reverse primers (5' GATCCTCTATGGTACTCGTGATGGCTTTAT 3') were added to a final concentration of 0.5 uM each. 10 ng of the *nahR/Psal* template DNA was added to each reaction and sterile dd$H_2O$ was added for a no template negative control. Two simultaneous PCR experiments were run, one containing 5X SuperFi™ GC Enhancer (Invitrogen) and the other without the enhancer. Temperature gradients were set up for the annealing temperature. The annealing temperatures used with the GC enhancer experiment were 56°C, 56.9°C, 58.1°C, and 59°C. The annealing temperatures used in the experiment with no enhancer were 56.9°C, 58.1°C, 59°C, 59.6°C. The initial denaturation step was at 98°C for 30 secs followed by 35 cycles of denaturation at 98°C for 10 seconds, annealing temperatures as stated above for 10 seconds, and extension at 72°C for 20 seconds. The final extension was performed at 72°C for 5 minutes and the products were stored at 4°C.

**DNA Gel electrophoresis**

All gels were made using 1% agarose in 1X Tris-Acetate EDTA (TAE) buffer. 2.5 µL RedSafe™ Nucleic Acid Staining Solution (iNtRON Biotechnology, Inc.) was added to each 50 mL gel for DNA visualization. 5 µL of 1 Kb Plus DNA Ladder (FroggaBio) was used as the ladder. 6X Gel Loading Dye Purple (New England BioLabs) or 10X BlueJuice™ Gel Loading Buffer (Invitrogen) were used for the loading dyes. The gels were run at 105V for ~45-60 minutes and imaged using the Gel Doc UV imager (BioRad).

**Gel extraction of *nahR/Psal* PCR products**

The gel extraction of *nahR/Psal* PCR products was done using the GeneJET Gel Extraction Kit (Thermo Scientific) as per the manufacturer's instructions. The DNA was eluted in 50 µL elution buffer and the concentration, A260/A280, A260/A230, and absorbance peaks were analyzed using the NanoDrop2000 spectrophotometer. The resulting purified PCR products were then run on a 1% agarose 1X TAE gel to confirm the correct size of the DNA fragment.

**Restriction enzyme digest of pSPPH21 and ligation with *nahR/Psal***

The digestion of pSPPH21 and ligation of the insert were performed in the same reaction using a blunt end cloning protocol adapted from Green and Sambrook (14). A vector:insert ratio of 1:3 was used by adding 45 fmol of pSPPH21 and 135 fmol of the isolated *nahR/Psal* PCR products in the first round of ligation. A second ligation was performed using phosphorylated IDT *nahR/Psal* gene fragment in addition to the isolated *nahR/Psal* PCR products (60 fmol vector:180 fmol insert). Phosphorylation of the IDT gene fragment was performed using T4 Polynucleotide Kinase (Thermo Scientific) as per the manufacturer's instructions. For the digestion/ligation reaction, in addition to the vector and insert, the reaction was set up with 3 U T4 DNA Ligase (Invitrogen), 1 mM riboATP (New England BioLabs), 1X NEBuffer r3.1 (New England BioLabs), 10 U NruI (New England BioLabs), and sterile dd$H_2O$ in a total volume of 20 ul. Negative control reactions were set up by adding sterile dd$H_2O$ in place of the DNA insert. The reaction was incubated at 37°C for 5 minutes, then at 25°C for 4 hours in a thermocycler. No heat-inactivation was performed and the resulting products were used in the transformation reactions.

**Heat shock transformation**

Ligated pSPPH21::*nahR/Psal*, empty pSPPH21 for the positive control, and NruI digested pSPPH21 for the negative control were transformed into Subcloning Efficiency™ DH5α Competent Cells (Invitrogen) and One Shot™ TOP10 Chemically Competent *E. coli* (Invitrogen) using heat shock transformation as per the manufacturer's instructions. In the first round of transformation, 7.8 ng of DNA was added to the cells. In the second round of transformation, 17.6 ng of DNA was added to the cells. After recovery in LB media at 37°C for one hour at 225 rpm, all cells were plated on LB agar plates containing 25 µg/mL chloramphenicol and grown at 37°C overnight. The resulting transformed colonies were grown overnight in LB broth containing 25 µg/mL chloramphenicol at 37°C at 225 rpm for plasmid isolation and screening.

**Plasmid isolation and quantification**

Plasmid isolation from overnight cultures was performed using the PureLink™ Quick Plasmid Miniprep Kit (Invitrogen), following the centrifuge protocol as per the manufacturer's instructions. The initial amount of overnight culture used was 1-2 mL. The plasmids were eluted in 75 µL TE Buffer and the concentration, A260/A280, A260/A230, and absorbance peaks were analyzed using the NanoDrop2000 spectrophotometer.

**Restriction enzyme digest for insert screening**

The isolated plasmids from the transformed colonies were digested with NruI (New England BioLabs) to screen for insertion of *nahR/Psal.* The restriction digest was carried out as per the manufacturer's instructions and the reaction was carried out at 37°C for 15 minutes. The products were then run on a 1% agarose 1X TAE gel along with undigested plasmid and empty pSPPH21 as negative controls.

**Restriction enzyme digest and re-transformation of clones 6 and 7**

After the initial screen, the isolated plasmids from clones 6 and 7 were re-digested with NruI (New England BioLabs) at room temperature for 24 hours. The resulting products were then re-transformed into One Shot™ TOP10 Chemically Competent *E. coli* (Invitrogen) following the same protocol as the second round of transformants described above. Two colonies from the clone 6 re-transformants and 4 colonies from the clone 7 re-transformants were chosen for further screening. The same protocol was followed for plasmid isolation, restriction digest, and gel electrophoresis.

**Induction and fluorescence assay**

Inductions were run in triplicate per colony at each concentration of salicylate. For each test, 1mL of overnight culture was induced with filter-sterilized salicylate (0, 1, 10, 50, 100, or 200 mM) for approximately 18 hours. Samples induced with 50mM salicylate or above did not grow, presumably due to toxicity, and were excluded from downstream analysis. The cells were harvested by centrifugation (10,000 r.p.m, for 3 minutes). The pellet was washed with 1 mL of phosphate-buffered saline (PBS) before being pelleted again by centrifugation (10,000 r.p.m, for 3 minutes). Cells were then resuspended in 200 µL of PBS for the fluorescence-based assay. Positive RFP and GFP fluorescing controls provided by Gawol *et al.* were induced in parallel with isopropyl β-d-1-thiogalactopyranoside (IPTG) as per Green & Sambrook protocol (D. Gawol, R. Floyd, K. Kohara, and Y. Lee, submitted for publication, 14).

In a black 96-well plate, 200 µL of the resuspended samples were plated. For RFP readings, the excitation wavelength was 580 nm, and the emission wavelength was 620 nm. For GFP readings, the excitation wavelength was 485 nm, and the emission wavelength was 528 nm. OD600 readings were also taken to estimate the cell growth stage and the total concentration of cells per well. For downstream analysis, fluorescence intensity was normalized by subtracting background PBS levels and dividing by OD600 to account for variations due to cell density.

**PET Bottle Induction**

A bottle of "Ruby Red Grapefruit Juice Drink" (Ocean Spray®) with a resin identification code of 1 - indicating PET (https://doi.org/10.1007/s41403-022-00324-4) was selected for detection of salicylate using the inducible biosensor. The PET bottle was emptied of its original contents and periodically filled with tap water and drained over a period of approximately 6 weeks prior to the experiment. It was then rinsed 3X with tap water and 3X with $ddH_2O$, before being filled with $ddH_2O$ - labeled "PET" (Fig. 6). Two clean glass bottles were autoclaved and one was rinsed with the rinsate from the PET bottle to account for the influence of the microbes present in the PET bottle on the experimental outcome - labeled "MICB Control" (Fig. 6). The other glass bottle was rinsed 3 X with tap water and 3 X with $ddH_2O$, before being filled with $ddH_2O$ - labeled " Negative Control" (Fig. 6). Autoclaving of the PET bottle was not performed in order to assess a possible breakdown scenario of PET in environmental conditions - with microbes present in tap water and in the surrounding environment allowed to grow. All 3 bottles were then left at room temperature and exposed to ambient sunlight for 6 days prior to the induction experiment. 111 µL of tap water from each storage condition was added to 1mL of LB with chloramphenicol and inoculated with our colony 1 biosensor. This induction step was performed in triplicate for each storage condition.

**Sanger sequencing**

Samples were prepared according to GeneWiz sample submission guidelines for pre-defined samples. Nanodrop results were used to assess the concentrations of plasmids purified by mini-prep and normalize all concentrations to 50 ng/µL in 10 µL. Where concentrations of the isolated plasmid were below 50 ng/µL - a volume containing 500 ng in total was submitted. Primers were submitted at a concentration of 5 µM each in 5 µL (Table 1).

**TABLE. 1 Primers used for sequencing.**

| Index | Name | Sequence (5'-3') |
|---|---|---|
| 1 | pSPPH21 F (Abrishamkar *et al.*) | CTTCGAATTCGCGGCCGCTTCTAG |
| 2 | pSPPH21 R (Abrishamkar *et al.*) | CTTCCTGCAGCGGCCGCTACTAGT |
| 3 | The_pSPPH21 F (Flanking BioBricks) | TGCCACCTGACGTCTAAGAA |
| 4 | The_ pSPPH21 R (Flanking BioBricks) | ATTACCGCCTTTGAGTGAGC |
| 5 | pT7ForP (Flanking NruI, F) | ATTTCGAACTCGTGACCGTT |
| 6 | pT7RevP (Flanking NruI, R) | ACTGACAGAAAATTTGTGCC |

The empty vector was sequenced using the primers published in Abrishamkar *et al.* flanking the RFP/GFP insertion, as well as primers annotated in SnapGene flanking the same region. The primers published in Abrishamkar *et al.* were homologous to the BioBricks prefix and suffix sequences on either side of the RFP/GFP insertion (P. Abrishamkar, H. M. Dana, S. Oveisi, and P. Tabassi, submitted for publication). These sequences share sufficient homology that the forward primer was identified as capable of binding both the forward and reverse sites in SnapGene software. The primers annotated in SnapGene (for pSB1C3, the vector on which pSPPH21 was built) bind outside of the prefix and suffix sequences, and were identified as having unique binding sites in SnapGene software.

The plasmid from colony one was sequenced with the primers flanking the BioBricks prefix and suffix sites, as well as primers flanking the NruI site which were lent from Agnew *et al.* All other colonies were sequenced using only the primers flanking the NruI site.
Sequencing data prior to the last unassigned base on the 5' end and following the first unassigned base at the 3' end of each read was not considered for analyses as they were expected to be unreliable (Fig. 7A). The model used for sequence read alignments for the transformed colonies was updated based on the sequencing results for the empty pSPPH21 vector in order to streamline the alignment process.

**Statistical analyses**

One-way ANOVA with Dunnett's multiple comparisons were used to evaluate differences in fluorescence intensity between samples that were induced and the uninduced controls. For all analyses, p-values $< 0.05$ were considered statistically significant. All statistics were computed using GraphPad Prism version 9.0.2 for Windows, GraphPad Software, Sandiego, California USA unless indicated otherwise.

## RESULTS

**PCR product size corresponds to the *nahR/Psal* gene fragment**

To test the functionality of inducible promoter detection by pSPPH21, the *nahR/Psal* gene fragment was used as a proof of concept test to be inserted into pSPPH21. The *nahR/Psal* gene block was PCR amplified to obtain copies of the insert for downstream ligation into pSPPH21. A temperature gradient PCR was performed to test the annealing temperatures of the primers. In addition, a GC enhancer provided with the DNA polymerase kit was added to half of the reactions. The amplification did not result in a single band but rather multiple bands suggesting non-specific binding. Comparing the two conditions, the reactions without the GC enhancer produced clearer and stronger bands at the expected size of the gene fragment, which is 1268 bp long. While there was a slight decrease in band intensity at roughly 750 bp, as the annealing temperature increased in the reactions without the GC enhancer, the temperature range was not high enough to eliminate background amplification. Both no template negative controls had amplification except faint bands around 100 bp which suggest the occurrence of primer dimers (Fig. 1).

To obtain the correct sized fragment, a gel extraction was performed to isolate the DNA contained within the four lanes without the GC enhancer around 1300 bp. The gel displays the products from the gel extraction with a single band around 1300 bp (Fig. 2).

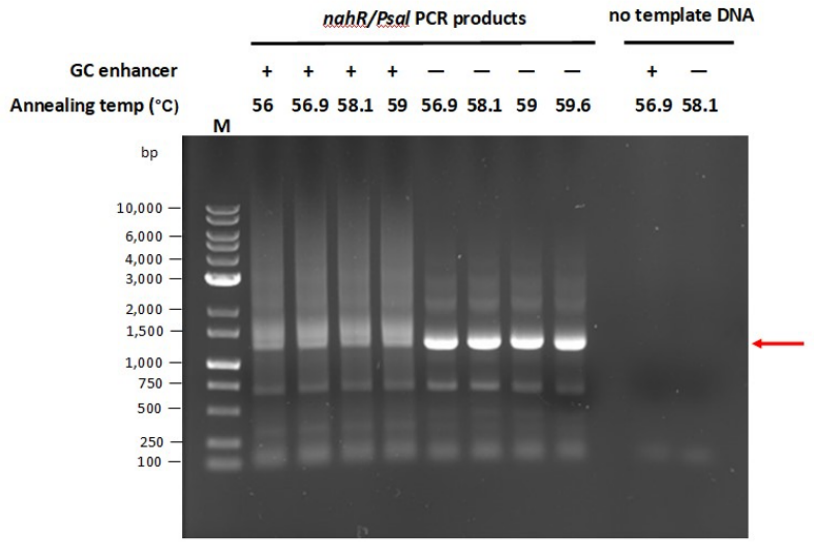


**FIG. 1 Amplification of *nahR/Psal* gene fragment.** Agarose gel electrophoresis (1% agarose) of amplified gradient PCR products using *nahR/Psal* primer sets and with or without 5X SuperFi™ GC Enhancer. Lanes: M = 1kb plus DNA size marker; 1-8 = *nahR/Psal* amplification; and 9-10 = no template negative control. Red arrow denotes expected band.

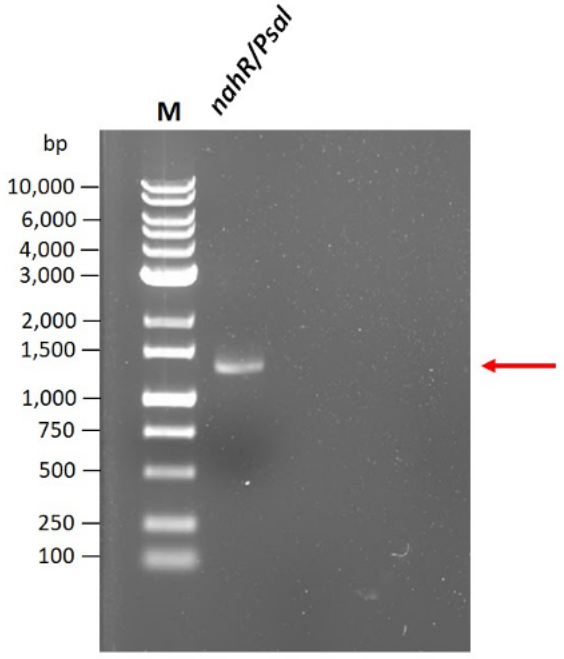


**FIG. 2 Gel electrophoresis of amplified *nahR/Psal* gel extraction product.** Agarose gel electrophoresis (1% agarose) with 1kb plus DNA size marker (Lane M) and *nahR/Psal* gel extraction product (Lane 1). Red arrow denotes expected band.

**Transformants contain pSPPH21 with potential insert**

After obtaining the *nahR/Psal* insert, we sought to ligate the insert into pSPPH21 and transform the recombinant plasmid into commercially competent DH5α *E. coli* cells. pSPPH21 has a single NruI site that allows for blunt ended digestion. The restriction enzyme, DNA ligase, vector and insert were added to a single reaction. Therefore, cut vector that is re-ligated without the insert would subsequently be re-digested by NruI, preventing circularized empty vector from being transformed into the cells. Once an insert is ligated into pSPPH21, the NruI site is destroyed and therefore can no longer be digested by NruI. Transformation of the digested and ligated plasmid yielded transformants in both DH5α and TOP10 *E. coli* cells. The negative control where no insert was added to the reaction yielded no colonies, confirming that NruI digestion of empty vector was effective. The positive control of undigested pSPPH21 yielded colonies as expected.

**A screen of isolated transformant plasmids suggest the *nahR/Psal* insert was ligated into pSPPH21**

13 transformant colonies were selected to investigate whether the *nahR/Psal* insert had ligated into the vector. After growing the colonies in overnight cultures, plasmids were isolated and screened using restriction enzyme digestion. The controls included stock pSPPH21 plasmid and pSPPH21 that had been transformed into commercially competent cells as a positive control, (Fig. 3). As stated above, the NruI site in pSPPH21 containing an insert would be destroyed upon successful ligation and therefore should show similar results to the undigested plasmid in a gel. Linearized pSPPH21 is expected to display a band around 3,520 bp, the length of the empty vector. The empty vector controls in the lanes where NruI was added behaved as expected with clear bands around 3,500 bp. The empty vector controls in the lanes where no NruI was added displayed a further migrated smear, likely supercoiled undigested plasmid, as well as a band higher than 10,000 bp, likely open circular or nicked conformation. The undigested samples behaved similarly to the undigested pSPPH21 control, suggesting a circularized plasmid. Colonies 1 and 13 where NruI had been added show similar smears on the gel as the undigested plasmids, indicating that the NruI site was destroyed. While colonies 6 and 7 displayed bright bands around 3,500 bp, there were also additional bands that were similar to their undigested controls. For this reason, we decided to re-digest the plasmids from colonies 6 and 7 overnight and then re-transform the products into TOP10 *E. coli* cells. The resulting transformant colonies were labeled C6.1-2 and C7.1-4. The screen of these plasmids indicated that the NruI site was destroyed in colonies 6.1-2 and 7.2-4 (Fig. 3).

**pSPPH21::*nahR/Psal* responds to salicylate induction**

The pSPPH21::*nahR/Psal* construct responds to salicylate, albeit moderately relative to a positive control. The signal from the Gawol *et al.* construct was roughly 50-fold greater than the maximum fluorescence observed from samples (D. Gawol, R. Floyd, K. Kohara, and Y. Lee, submitted for publication). However, results did show an elevated response in colonies 1 and 6.1 treated with 10mM salicylate relative to their respective uninduced controls ($p<0.05$). Furthermore, this response appeared to be concentration-dependent given the same colonies induced with 1mM salicylate elicited fluorescence levels comparable to controls. Notably, colony 13 alone did not respond to any salicylate concentration. In addition, when responses were detected, they were inconsistent between the different colonies and within colony replicates. First, colony 1 had an elevated fluorescent response compared to the other colonies tested at each concentration of salicylate. Interestingly, this was also true of the uninduced control which implies different levels of leaky expression between colonies. Even amongst replicates, variation in fluorescence intensity was observed for colonies 6.1 and 7.3 as shown by large error bars (Fig. 4).

**Fluorescence is not observed by salicylate alone or the empty pSPPH21 vector induced with salicylate**

To evaluate if the empty pSPPH21 vector was responsible for the observed fluorescence, a secondary assay was run with the empty vector and colony 1. Excluding 10mM salicylate, colony 1 had elevated fluorescence relative to the empty vector at all concentrations ($p<0.001$). At 10mM salicylate induction, two of three replicates responded with a higher fluorescence intensity than the empty vector. The other replicate had minimal fluorescence, comparable to the empty vector, and likely accounts for the diminished statistical difference (Fig. 5). Additionally, fluorescence was not detected against salicylate alone which had fluorescence readings comparable to PBS (data not shown). Overall, this suggests that the pSPPH21::*nahR/Psal* construct responds uniquely to salicylate induction.

**PET breakdown due to UV light is not detectable by the *nahR/Psal* biosensor after 6 days**

The *nahR/Psal* biosensor could not detect significant differences in induction between the plastic and glass bottles, or between glass bottles hosting different populations of microbes (Fig. 6). All bottles were incubated for 6 days prior to induction.

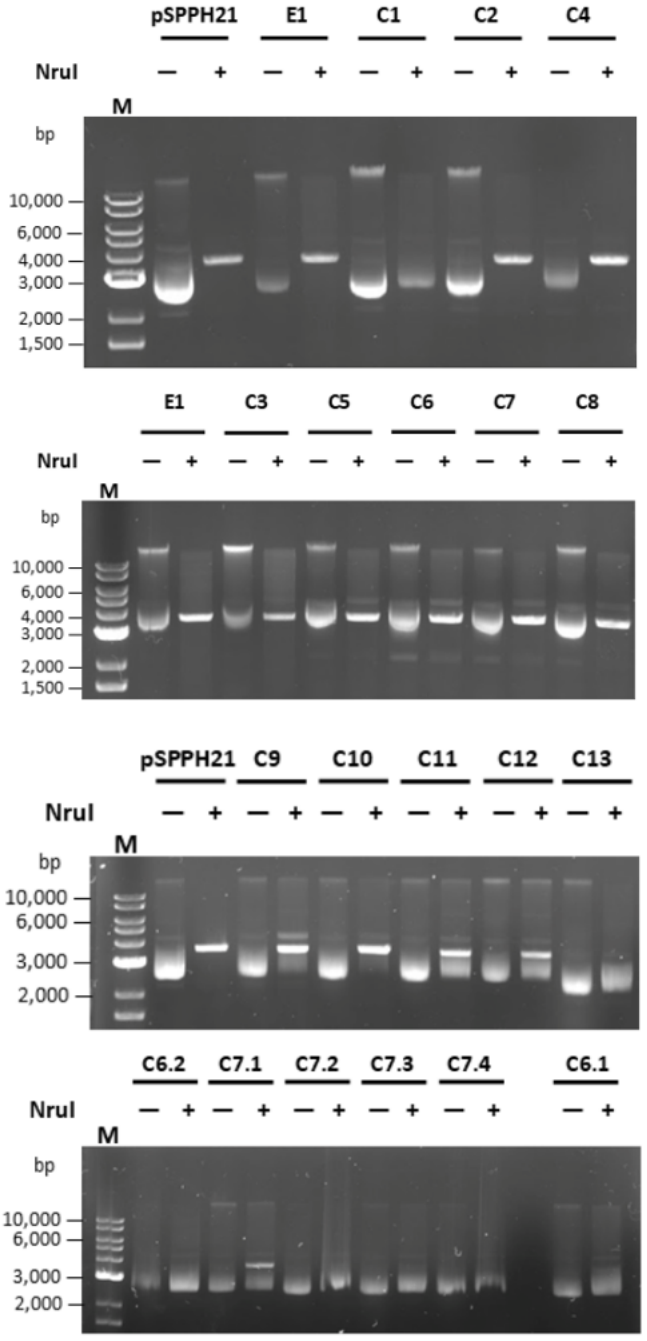


**FIG. 3 Gel electrophoresis of plasmids treated with or without NruI.** Agarose gel electrophoresis (1% agarose) with 1kb plus DNA size marker (Lane M), stock pSPPH21 plasmid (pSPPH21), transformed pSPPH21 vector without insert (E1), and tested colonies as labeled (C#).

**The empty vector is as expected, with RBS sequences associated with both GFP and RFP**

The empty vector sequence was almost identical to the model we were provided by the MICB 401 teaching team. The provided model contains a chloramphenicol resistance gene with an associated lambda t0 terminator and an origin of replication, though this region was not sequenced in its entirety. The NruI restriction site is flanked by RFP and GFP in opposing orientations, each with an associated RBS. The RBS site upstream of GFP is not the one which was annotated in the model. However, it is still present 8 base pairs upstream of the start codon for GFP, and is a BioBrick registered in the iGEM Registry of Standard Biological Parts as BBa_K1725317 (http://parts.igem.org/wiki/index.php/Part:BBa_K1725317). The BioBrick RBS is flanked by two BioBrick cloning scars registered as BBa_J70032 in the iGEM registry (http://parts.igem.org/Part:BBa_J70032). RFP and GFP are followed by the

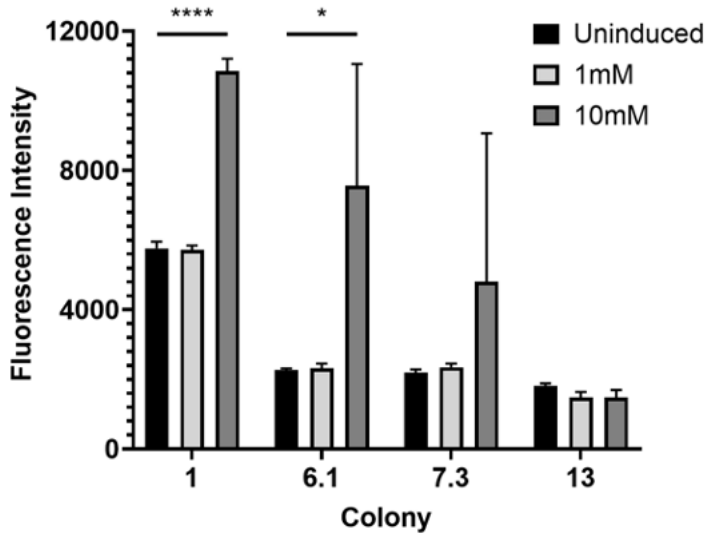


**FIG. 4 *nahR/Psal* biosensor responds to salicylate induction.** Colonies were treated with 0, 1, or 10mM of salicylate for 18 hours before being resuspended in PBS. Fluorescence intensity reflects reads normalized by cell density, taken at an excitation wavelength of 485 nm and emission wavelength of 528 nm. n=3 per condition. Mean +/- SD. Statistical significance was determined by comparing each group to the uninduced control via one-way ANOVA with Dunnett's multiple comparisons (* p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001).

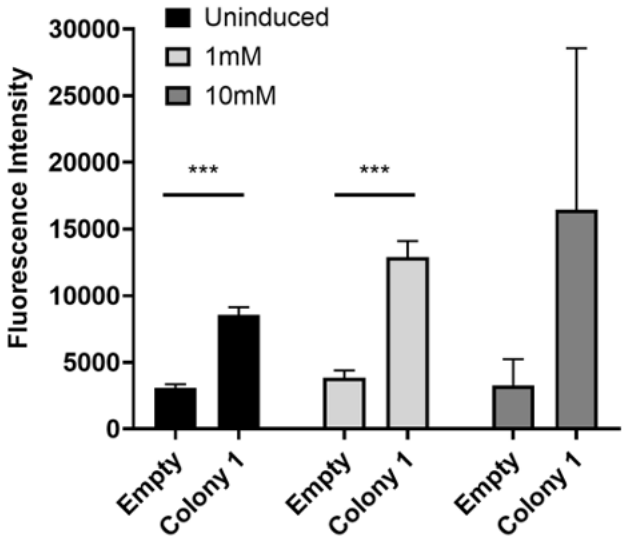


**FIG. 5 Colony 1 responds to salicylate induction, uniquely to the empty pSPPH21 vector.** Colonies were treated with 0, 1, or 10mM of salicylate for 18 hours before being resuspended in PBS. Fluorescence intensity reflects reads normalized by cell density, taken at an excitation wavelength of 485 nm and emission wavelength of 528 nm. n=3 per condition. Mean +/- SD. Statistical significance was determined by comparing each group to the uninduced control via an unpaired t-test (* p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001).

standard BioBrick prefix and suffix, respectively and have associated terminator sequences. Aside from the discrepancy surrounding the RBS, no mismatches were present between the model and the sequencing reads prior to the first unassigned base in each read.

**All selected colonies plasmids have deletions within or surrounding the NruI site, without the *nahR/Psal* fragment insertion**

The majority of colonies identified in NruI restriction digest screening show the same mutation. Colonies 1, 6.1-6.2, and 7.2-7.4 (6 in total) all show a two base pair deletion of "GC" within the NruI site (Fig. 7B). This result was consistent between forward and reverse reads, as well as both primer sets used for the colony 1 plasmid. Colony 13 was the only deviation from this genotype, with a 433 base pair deletion spanning the NruI site, as well as the RBS sites associated with each fluorescent protein, the first 20 base pairs of the GFP gene and first 357 base pairs of the RFP gene (Fig. 7C). This result was obtained from a single read with a single primer, as the forward primer (Primer 5 - Table 1) did not bind the template. This lack of binding is consistent with the sequencing results from the reverse primer - since the primer was designed to bind within the putative deleted region. The single read from the

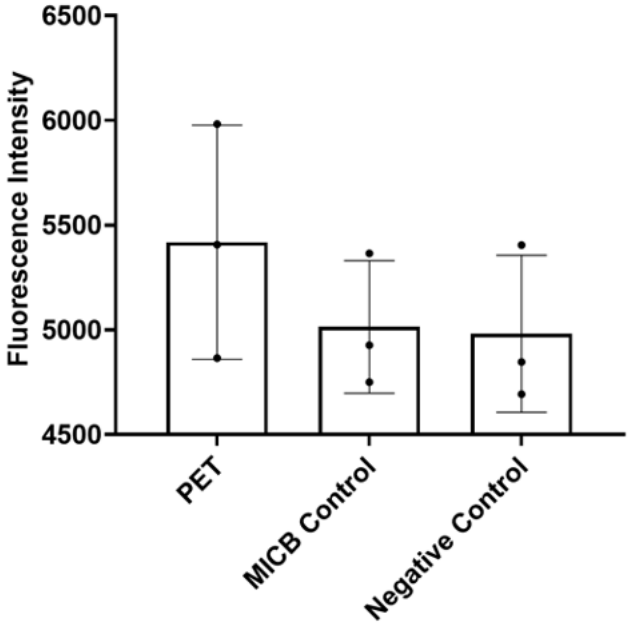


**FIG. 6 *nahR/Psal* biosensor did not detect differences in salicylate levels.** Fluorescence intensity reflects reads normalized by cell density, taken at an excitation wavelength of 485 nm and emission wavelength of 528 nm. n=3 per condition. Mean +/- SD. Statistical significance was tested by comparing each group via one-way ANOVA with Dunnett's multiple comparisons (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$). Conditions: PET: $ddH_2O$ stored in polyethylene terephthalate bottle. MICB Control: $ddH_2O$ stored in autoclaved glass bottle rinsed with rinsate from PET bottle. Negative Control: $ddH_2O$ stored in autoclaved glass bottle.

colony 13 plasmid did confirm the presence of the first half of the chloramphenicol resistance gene with 100 percent identity to the provided plasmid model.

## DISCUSSION

The *nahR/Psal* fragment was amplified, and its expected size was verified by gel electrophoresis. Combined digestion and ligation of *nahR/Psal* with the pSPPH21 vector followed by heat shock transformation of *E.coli* yielded transformants, while the same treatment applied to pSPPH21 alone yielded no transformants. The simplest explanation for this result, when taken in isolation, is that the fragment was ligated into the vector, allowing it to circularize while at the same time disrupting the NruI site by splitting it into two halves on either side of the insert.

The sequencing results contradicted this explanation and showed repeated loss of NruI, and in the majority (6/7) of cases this was due to an identical 2 base pair deletion within the restriction site, corresponding to one base pair deleted on either side of the cut site. In one case the site was lost through deletion of a large region including the site itself and portions of both *RFP* and *GFP*. This indicates a strong selective pressure against the presence of the NruI site in the plasmid - which is expected in the combined digestion/ligation reaction conditions used. Any plasmid containing the restriction site can be cleaved, yielding a linear fragment that will not replicate within cells that are transformed with it, unless re-ligated within the cell (15). The sequencing results suggest a mechanism of NruI loss that involves the deletion of regions on either side of the cut site. Since ligation of the insert was presumably another possible route for NruI loss, the insertion was either less efficient than the deletion route, or the insertion route was strongly selected against. The *nahR/Psal* PCR product was not sequenced, but the mechanism of ligation is not sequence dependent and any double stranded DNA present in the reaction mixture could have ligated into the vector (16).

Fluorescence was detected at the expected excitation and emission profile for GFP, and generally increased in response to salicylate treatments for the colony 1 (DH5α) obtained following digestion/ligation and transformation. Colonies 6 (TOP10) and 7 (TOP10) showed variable induction results, but some replicates did show high levels of fluorescence when treated with salicylate. Colony 13 (DH5α), which had deletions of the RBS for both RFP and GFP in pSPPH21, showed very low levels of fluorescence, similar to *E.coli* DH5α containing the empty pSPPH21 vector. Crucially this fluorescence did not vary with salicylate concentration in the cultures with the empty vector or the vector with the deletion. Colony 13 and the culture containing the empty vector were not treated within a single experiment, however, and so the difference in their basal expression rates cannot be directly addressed.

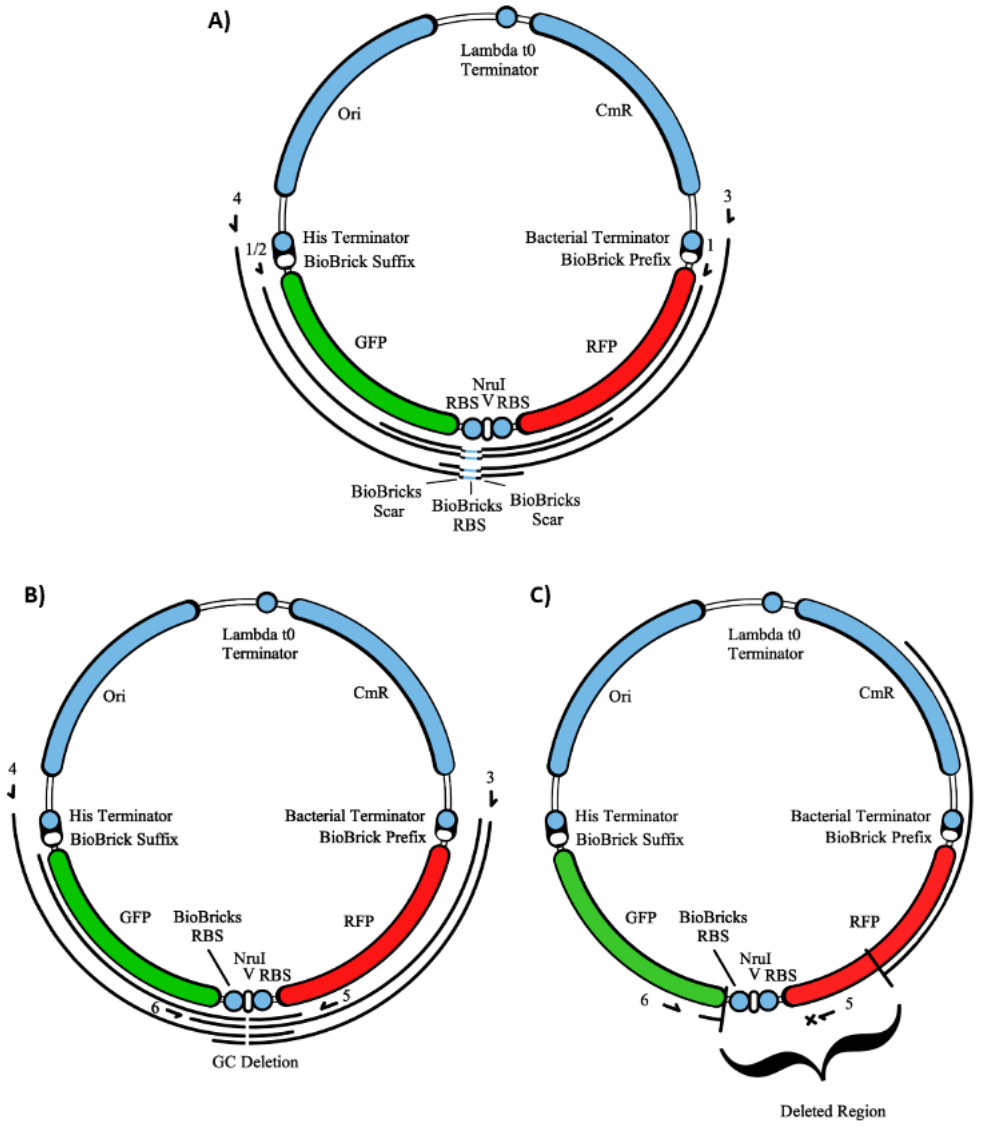


**FIG. 7 Sequences for the empty pSPPH21 vector and colonies responsive to salicylate. (A)** pSPPH21 prior to transformation. **(B)** Colonies 1, 6.1-6.2, and 7.2-7.4, which show a two base pair deletion within the NruI site. **(C)** Colony 13, which has a deletion spanning the NruI site. Panels A and B are aligned to an updated plasmid model incorporating the analogous BioBricks RBS upstream of GFP. Primers used for sequencing are annotated by the indices listed in Table 1. Acronyms: Ori = origin of replication; CmR = chloramphenicol resistance gene; RBS = ribosome binding site; RFP/GFP = Red/Green fluorescent protein.

The variation in salicylate response between colony 1 and colonies 6 and 7 may be due to genomic differences between DH5α and TOP10. The genomic sequence of TOP10 is not published. However, it is most closely related to DH10B according to ThermoFisher product information. DH10B itself is derived from *E.coli* K-12 strain MG1655 (17). Sequencing of the entire plasmid could reveal the insertion of *nahR/Psal* into an alternate site in the plasmid - however, the presence of NruI, its absence from any other location in the plasmid, and the mutations observed at the site provide strong evidence that any insertion element would integrate at that point instead of elsewhere on the plasmid. Since blunt ended ligation is known to be less efficient than other types of ligation, an insufficient number of clones may have been screened to obtain transformants (18). However, this does not explain the inducible gene expression seen in colony 1.

The salicylate used was of unknown age, since no date was printed on the label (Allied Chemical & Dye Corporation). The company has since undergone name changes and merged

with Honeywell, suggesting that the salicylate may have been produced at least 30 years ago (https://www.honeywell.com/us/en/company/our-history). Due to the potential age of the product and its light sensitive properties, it is plausible that the salicylate was partially degraded. This would explain the weak fluorescent signals induced at relatively high concentrations. Salicylate was toxic at most at 50 mM in the experimental conditions, resulting in no visible growth in overnight cultures. This toxicity could be due to the salicylate itself, or potential breakdown products as salicylate is vulnerable to breakdown when exposed to light (19,20). This could result in disproportionate cell death, which would increase the $OD_{600}$ readings and therefore decrease the observed fluorescence response during normalization, masking higher expression of GFP in the surviving cells. Salicylate can also induce persister cell formation, and induce general drug resistance through reductions in membrane permeability, masking the impact of antibiotics - this would also increase the proportion of cells without the plasmid of interest, and mask increases in GFP expression in the assay (21). Salicylate itself has no absorption or emission in the ranges tested for the 96-well plate assay (22). The possibility of breakdown products of salicylate or contaminants in the stock producing fluorescence was also assessed, and no fluorescence beyond background levels was found.

Fluorescence did not increase with exposure to water stored in a PET container. PET is known to break down and release benzoate under UV radiation (23). Benzoate, salicyl alcohol, and methyl salicylate are all found as contaminants in PET bottles - but are not themselves inducers of nahR (24). In order to test the hypothesis that these contaminants could be metabolized into salicylate by environmental microbes, induction of colony 1 was performed. This experiment did not result in any significant induction of gene expression after 6 days. Longer incubation times may result in a higher level of metabolized contaminants.

Selective pressure against the insertion could be conferred by the NahR repressor protein binding to and preventing expression of GFP, but also downstream sequences such as the chloramphenicol resistance gene. This would provide strong selective pressure against plasmids containing the insert in the presence of chloramphenicol. However, the chloramphenicol resistance gene has its own promoter, and its regulation should be independent of a promoter at that distance (25). However, Gawol *et al.* found that insertion of the *lacI* gene and its corresponding inducible promoter were able to ligate into the plasmid without causing cell fatality in chloramphenicol (D. Gawol, R. Floyd, K. Kohara, and Y. Lee, submitted for publication).

**Conclusions** No explanation for the fluorescence resulting from salicylate treatment on the putative transformant colonies arises from the fluorescence of the salicylate itself, or the *E.coli* DH5α containing pSPPH21. The inducible fluorescence of the colonies was only observed in those containing a GC deletion in pSPPH21. This genotype has no predicted promoter elements capable of driving GFP expression. Two distinct possibilities remain that account for the presence of the salicylate inducible expression of GFP. *GFP*, along with the *nahR/Psal* biosensor system, was integrated into the genome of *E.coli*, or the biosensor was integrated into pSPPH21 and this recombinant plasmid is present in the identified colonies, but at a minority relative to the pSPPH21 GC deletions. This would account for the inducible fluorescence phenotype, while also accounting for the sequencing results. Both genomic integration and sub-populations of transformant plasmid remain unlikely, and further investigation is needed to determine the presence or absence of the *nahR/Psal* biosensor system and *GFP* within the putative transformant cells.

**Future Directions** In order to screen for potential pSPPH21 plasmid containing the insert of interest, primers spanning the junction between the ends of *nahR/Psal* and the pSPPH21 plasmid in the insert region could be designed to amplify purified plasmid from colony 1. If a sub-population of plasmids containing the insert exist, the primers would hybridize with that specific junction and amplify only the recombinant plasmid. The same primers spanning the junction could be used for overlap PCR, with the amplified *nahR/Psal* fragment and pSPPH21 as templates, bypassing the inefficient blunt ended ligation step entirely (26). Either

of these products could then be used for a further heat shock transformation to isolate transformants with only the plasmid of interest.

Considering the inefficiency of blunt ended ligation in this condition, several methods of optimization for blunt ended ligation could be employed. Polyethylene glycol can increase the efficiency of blunt ended cloning reactions (27). NEB offers ligase meant to be specific for blunt-ended ligations. Introducing a kanamycin or similar antibiotic resistance gene to the insert and screening for colonies that can grow on both chloramphenicol and kanamycin could reduce the burden on screening for transformants - but would require the extra step of introducing the additional antibiotic resistance gene.

Desired insertion elements could also be amplified with Taq polymerase, resulting in triple-A overhangs. This could then be combined with pSPPH21 cut with NruI and treated with a terminal transferase to add complementary single T overhangs. These two ends would base pair and could then be joined by ligase to form the recombinant insert, in a process known as TA cloning (28).

None of these methods would be suitable if pSPPH21 is to be used as a screening platform for inducible promoters in environmental DNA. Overlap PCR requires knowledge of the sequence to be inserted, blunt ended ligation is too inefficient to be practical, double antibiotic selection neatly combines the problems with the previous two methods. TA cloning would require sequence knowledge for primer design as well.

Point mutation of the NruI site to a sticky-ended restriction site by PCR mediated site directed mutagenesis would be a convenient option (29). This would allow screening by digestion of both the plasmid and environmental DNA samples with a sticky-ended restriction enzymes and efficient ligation. This process would also be a-directional, but would introduce some bias due to the relative prevalence of the restriction site in different parts of the genome of the environmental strains being tested.

## ACKNOWLEDGEMENTS

We would like to thank Dr. David Oliver, Aditi Nallan, and Jade Muileboom for their guidance and technical support throughout this project. In addition, a special thanks to fellow classmates working on parallel SIGEX projects for their donation of recombinant pSPPH21 used as our positive controls as well as general scholarly advice. This project was funded by the Department of Microbiology and Immunology at the University of British Columbia.

## CONTRIBUTIONS

KF, KM, and TN worked together on the experiments and reviewed the manuscript.
KF contributed to the abstract, introduction (editing), materials and methods (induction and fluorescence assay, statistical analyses), the results (sections relating to fluorescence and salicylate induction), and figure legends. Performed the statistical analyses and created figures 4-6 on GraphPad.
KM contributed to the title, introduction, the materials and methods section, (PET bottle and Sanger sequencing) and the results section (PET bottle and Sanger sequencing). Also contributed to the discussion and future directions sections. Performed sequencing alignments and analysis in SnapGene software and generated Sanger sequencing results images using FireAlpaca graphic design software.
TN did the first nine sections of the materials and methods (bacterial strains and plasmids, PCR amplification, gel electrophoresis and extraction, digestion/ligation, transformation, plasmid isolation and screening), formatted the gel electrophoresis images, wrote the results sections for the PCR products, transformation, and screening, and contributed ideas to the future directions.

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