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AraC-Based Biosensor for the Detection of Isoprene in *E. coli*

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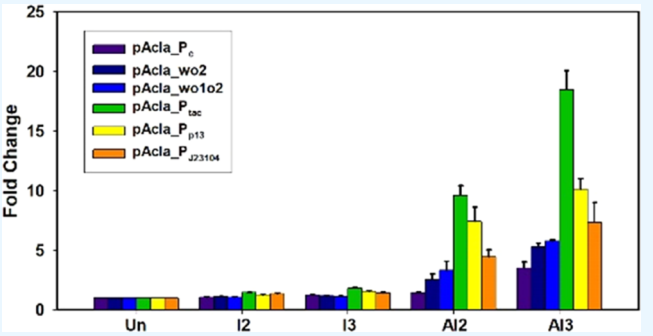
ABSTRACT: Isoprene is a valuable platform chemical, which is produced by engineered microorganisms, albeit in low quantities. The amount of isoprene produced is usually measured by gas chromatography, which can be time-consuming and expensive. Alternatively, biosensors have evolved as a powerful tool for real-time high-throughput screening and monitoring of product synthesis. The AraC-pBAD-inducible system has been widely studied, evolved, and engineered to develop biosensors for small molecules. In our preliminary studies, the AraC-pBAD system was mildly induced at higher isoprene concentrations when arabinose was also available. Hence, in the present study, we designed and constructed a synthetic biosensor based on the AraC-pBAD system, wherein the ligand-binding domain of AraC was replaced with IsoA. On introducing this chimeric AraC-IsoA (AcIa) transcription factor with the native P_{BAD} promoter system regulating *rfp* gene expression, fluorescence output was observed only when wild-type *Escherichia coli* cells were induced with both isoprene and arabinose. The biosensor sensitivity and dynamic range were further enhanced by removing operator sequences and by substituting the native promoter (P_{AraC}) with the strong tac promoter (P_{tac}). The chimeric sensor did not work in AraC knockout strains; however, functionality was restored by reintroducing AraC. Hence, AraC is essential for the functioning of our biosensor, while AcIa provides enhanced sensitivity and specificity for isoprene. However, insights into how AraC-AcIa interacts and the possible working mechanism remain to be explored. This study provides a prototype for developing chimeric AraC-based biosensors with proteins devoid of known dimerizing domains and opens a new avenue for further study and exploration.



1. INTRODUCTION

Isoprenoids and terpenoids form a diverse array of secondary metabolites, which have tremendous industrial application. Isoprene is a hemiterpene with application in the production of synthetic rubber, biofuels, and elastomeric materials.[1,2] Isoprene is produced either through the mevalonate pathway (MVA) found in eukaryotic systems including plants[3−5] or through the methyl-erythritol phosphate (MEP) pathway reported in prokaryotes.[6,7] Plants are the highest natural producers of isoprene; however, the volatile nature of isoprene makes the detection, quantification, and extraction from plants very tedious for commercial applicability. For industrially sustainable production, metabolic engineering of microorganisms, especially *Escherichia. coli*, has been widely explored and undertaken. Various approaches including expression of a few heterologous genes to the introduction of the complete MVA pathway have been undertaken to increase the isoprene production levels in *E. coli*.[8−10] By using other more recent strategies such as protein engineering,[11] directed evolution,[12] and combinatorial part libraries,[13] large mutant libraries of isoprene producers can be made. However, screening of such libraries of strains for production of isoprene through a gas chromatograph is tedious, expensive, and time-consuming.

Biosensors for detection of small molecules have been widely developed and applied for high-throughput screening and real-time monitoring of intracellular levels.[14−17] Biosensors based on transcription factors, fluorescent or chimeric proteins, RNA, two-component systems, etc. have been developed and studied extensively. Transcription factor (TF)-based biosensors are composed of a metabolite-responsive inducible gene expression system linked to a reporter gene, commonly a fluorescent or luminescent gene.[18−21] By coupling metabolite recognition with changes in the reporter gene expression (fluorescent/luminescent), these TF-based biosensors allow detection and quantification of intracellular metabolites in a concentration-dependent sensitive manner through rapid, high-throughput techniques such as fluorescence/luminescence-based assays and FACS. TF biosensors have been engineered and repurposed for sensing metabolites such as

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mevalonate, 3-hydroxypropionic acid, isopentenyl pyrophosphate (IPP), itaconic acid, etc.[21−25]

The AraC-pBAD system is a widely studied and used inducible expression system.[26,27] In the absence of L-arabinose, AraC forms homodimers, which bind to the operator O2 and I1 half-sites to block transcription by preventing RNA polymerase from binding to the promoter. L-Arabinose binds to the ligand-binding domain (LDB) of AraC, causing a conformational switch in the orientation of the DNA-binding domain (DBD). Consequently, the binding of AraC dimers to the promoter I1 and I2 half-sites is energetically favored, thereby promoting transcription.[27,28] The functionality of this system has been expanded by evolving AraC to recognize a range of other metabolites such as D-arabinose,[29] mevalonate,[24] ectoine,[30] triacetic acid lactone,[31] etc. Additionally, the ability of AraC to adopt to two conformational states has been exploited for developing TF-based biosensors for the detection of isopentenyl pyrophosphate (IPP).[25] In this study, the ligand-binding domain (LBD) of *araC* was replaced with the *idi* gene, which is also known to dimerize. In another study following a similar approach, the AraC-pBAD system was engineered to make it respond to light instead of arabinose by replacing the LBD of AraC with light-triggered dimerization domains (i.e., VVD, LOV, VfAu1).[32]

A previous report on the development of a biosensor for isoprene has been found.[33] The biosensor was based on the TbuT regulator, which controls the toluene−benzene utilization pathway. Since isoprene is a less-preferred ligand for this regulator, the authors improved the sensitivity by developing a transcription cascade using T7 RNA polymerase. In this study, we developed a biosensor for isoprene based on the AraC-pBAD system. The LBD of AraC was swapped with IsoA, which is known to form the active site of the isoprene−mono-oxygenase complex involved in isoprene metabolism in *Rhodococcus sp*. AD45.[34,35] To improve the sensitivity, variants of this sensor were made by changing the native promoter (P_c or P_{araC}) and removing the operator sequences O1 and O2. Here, we report a biosensor with improved sensitivity and specificity for isoprene and provide a prototype for developing chimeric AraC transcription factors with proteins devoid of dimerizing domains.

2. MATERIALS AND METHODS

2.1. Bacterial Strains and Growth Conditions. *E. coli* DH5α cells were used for routine cloning, plasmid propagation, and maintenance, while *E. coli* K-12 MG1655 and its mutant strains were used for the biosensor experiments. *E. coli* MG1655 ΔAraC (*E. coli* AB1655) and *E. coli* MG1655 ΔAraC ΔAraBAD (*E. coli* AB1656) (Table 1) were a kind gift from Prof. Mustafa Hani Khammash (ETH Zurich).[32] Routine cultivation of bacterial strains was carried out in LB-Miller broth (with or without antibiotic), in a shaking incubator at 180 rpm and 37 °C. For biosensor studies and fluorescence assays, cultures were grown in M9 minimal medium (Himedia) supplemented with 0.24 g L^{-1} $MgSO_4$, 0.011 g L^{-1} $CaCl_2$, 0.5 μg L^{-1} thiamine, 4 g L^{-1} glucose, and 40 μg L^{-1} chloramphenicol, in a shaking incubator at 180 rpm, maintained at 30 °C. Biosensor assays were also undertaken in tryptone broth (tryptone = 10 g L^{-1}, NaCl = 5 g L^{-1}, NaOH = 1 mM)[32] and M9 minimal medium supplemented with 5 g L^{-1} yeast extract.

2.2. Standard Procedures and Reagents for Cloning. Oligonucleotide primers were synthesized by Eurofins and Sigma-Aldrich (Table S1). Plasmid DNA was extracted from overnight cultures of transformed cells using the FavorPrep plasmid DNA extraction mini-kit (Favorgen Biotech Corp). Polymerase chain reaction (PCR) amplification for cloning was carried out using Thermo Scientific Phusion high-fidelity DNA polymerase (Thermo Fisher Scientific) following the manufacturer's protocol. Restriction enzymes from New England Biolabs and Thermo Fisher Scientific were used with their recommended buffers for restriction-based cloning. DNA purification and gel extraction were carried out using the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel).

Table 1. List of Bacterial Strains and Plasmids Used in this Study

bacterial strains		
	description	refs
E. coli DH5α	F- Φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17(rk−, mk+) *pho*A *sup*E44 *thi*-1 *gyr*A96 *rel*A1 λ-	laboratory stock
E. coli K-12 MG1655	F- lambda- *ilv*G- *rfb*-50 *rph*-1	laboratory stock
E. coli MG1655_KO	AraC knockout mutant of *E. coli* K-12 MG1655 strain	this study
E. coli AB1655	MG1655, K-12 ΔaraC	32
E. coli AB1656	MG1655, K-12 ΔaraC ΔaraBAD	32
plasmids		
	description	refs
pEH006	Chl^r; P_{araC}-*araC*-T_{rrnB1} and P_{araBAD} -T7sl-EcRBS-*rfp*-T_{dbl}	13
pSA_MP	Chl^r; Master plasmid with EH006 backbone, T_{rrnB2}-P_{tac}-*rfp*-T_{dbl}	this study
pSA_BAD_rfp	Chl^r; P_{araC}-*araC*-T_{rrnB2} and P_{araBAD} -*rfp*-T_{dbl}	this study
pSA_Rha_rfp	Chl^r; P_{rhaSR}-*rhaSR*-T_{rrnB2} and P_{rhaB} -*rfp*-T_{dbl}	this study
pAcIa_P_c	Chl^r; P_{araC}-*AcIa*-T_{rrnB2} and P_{araBAD} -*rfp*-T_{dbl}	this study
pAcIa_control1	Chl^r; P_{araC} -T_{rrnB2} and P_{araBAD} -*rfp*-T_{dbl}	this study
pAcIa_wO2	Chl^r; P_{araC}-*AcIa*-T_{rrnB2} and P_{araBAD} (without O2)-*rfp*-T_{dbl}	this study
pAcIa_wO1O2	Chl^r; P_{araC}-*AcIa*-T_{rrnB2} and P_{araBAD} (without O1-O2)-*rfp*-T_{dbl}	this study
pAcIa_P_{tac}	Chl^r; P_{tac}-*AcIa*-T_{rrnB2} and P_{araBAD} (without O1-O2)-*rfp*-T_{dbl}	this study
pAcIa_P_{p13}	Chl^r; P_{p13}-*AcIa*-T_{rrnB2} and P_{araBAD} (without O1-O2)-*rfp*-T_{dbl}	this study
pAcIa_P_{J23104}	Chl^r; P_{J23104}-*AcIa*-T_{rrnB2} and P_{araBAD} (without O1-O2)-*rfp*-T_{dbl}	this study
pRha_ISP_AcIa_P_c	Chl^r; P_{rhaSR}-*rhaSR*-T_{rrnB2} and P_{rhaB}-*ispS*-T_{b1002}; P_{araC}-*AcIa*-T_{b1002} and P_{araBAD} -*rfp*-T_{dbl}	this study
pRha_ISP_AcIa_P_{tac}	Chl^r; P_{rhaSR}-*rhaSR*-T_{rrnB2} and P_{rhaB}-*ispS*-T_{b1002}; P_{tac}-*AcIa*-T_{b1002} and P_{araBAD} (without O1-O2)-*rfp*-T_{dbl}	this study
P_C_AraC_AcIa_P_{tac}	Chl^r; P_{araC}-*AraC*-T_{rrnB2} and P_{tac}-*AcIa*-T_{b1002} and P_{araBAD} (without O1-O2)-*rfp*-T_{dbl}	this study
pCAS	Kan^r; repA101(Ts); P_{cas}-cas9; P_{araB}-Red; *lacI*^{q} P_{trc}-sgRNA-*pMB1*	37
pTargetF	Spec^r; *pMB1 aadA* sgRNA-*cadA*	37
ptargetF_g1	Spec^r; *pMB1 aadA* sgRNA-*g1* gRNA_1 seq- CAGAATCACTGCCAAAATCG	this study
pTargetF_g2	Spec^r; *pMB1 aadA* sgRNA-*g2* gRNA_2 seq- ATATAACCTTTCATTCCCAG	this study
pTargetF_g1_HA	Spec^r; *pMB1 aadA* sgRNA-*g1* Δ*araC*	this study
pTargetF_g2_HA	Spec^r; *pMB1 aadA* sgRNA-*g2* Δ*araC*	this study

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Ligation was done using T4 DNA Ligase (Invitrogen). For infusion cloning, an In-Fusion HD cloning kit (Takara Bio) was used following the recommended protocol. Chemically competent *E. coli* cells were prepared by the Inoue method,[36] and cells after heat shock were revived with SOC medium. Colony PCR and routine PCR confirmation of plasmids were done using Taq 2X Master Mix (New England Biolabs). All plasmid constructs and strains (listed in Table 1) were sequence-verified (Eurofins Genomics India Pvt. Ltd.).

2.3. Construction of Plasmids. *2.3.1. Construction of Plasmid SA_MP.* An equimolar concentration of MP_f and MP_r was mixed in 1× ligation buffer, heated to 95 °C for 5 min, and allowed to slowly cool to room temperature to anneal. This annealed double-stranded oligonucleotide carrying the tac promoter (P_{tac}) was cloned into pEH006 (a kind gift from Dr. Naglis Malys, University of Nottingham)[13] using *Asc*I and *Nde*I restriction sites to form plasmid pSA_MP.

2.3.2. Construction of Biosensor Plasmids. Chimeric AraC, henceforth referred to as AcIa, was designed by swapping the LBD sequence of *araC* with the complete nucleotide sequence of the *isoA* gene. The AcIa sequence (sequence given in the Supporting Information) along with the other native components of the pBAD expression system was synthesized by GenScript Biotech Corporation. Oligonucleotide primers, AraC_f and pBAD_r, were used to PCR-amplify the synthesized AcIa-pBAD fragment, which was subsequently cloned into pSA_MP upstream to the *rfp* reporter gene using the restriction sites *Eco*RI and *Spe*I to get the biosensor plasmid pAcIa_P_c (Figure S1). Plasmid pAcIa_P_c was digested with *Eco*RI and *Mfe*I, and the backbone without the AcIa was religated to generate the control plasmid, pAcIa_control1. The construct pSA_BAD_rfp was generated by amplifying the arabinose-inducible P_{BAD} promoter using the primer pairs AraC_f and pBAD_r with *E. coli* MG1655 genomic DNA as the template and introducing it in pSA_MP at the *Eco*RI and *Spe*I restriction sites.

The variant plasmid pAcIa_wO2 does not have the operator sequence O2, while pAcIa_wO1O2 does not have both operator sequences (O1 and O2). pAcIa_wO2 was constructed by overlap-extension PCR of fragments amplified using AraC_f and wO2_r and wO2_f and pBAD_r followed by cloning into pSA_MP using *Eco*RI and *Spe*I restriction sites. Similarly, pAcIa_wO1O2 was constructed by overlap-extension PCR of fragments amplified using primers AraC_f and wO2_r and wO2O1_f and pBAD_r.

The native promoter (P_c or P_{AraC}) of AraC in the pBAD expression system was replaced by P_{tac}, P_{p13}, and P_{J23104} in the constructs pAcIa_P_{tac}, pAcIa_P_{p13}, and pAcIa_P_{J23104}, respectively, following the same strategy used for pAcIa_wO1O2, using primers tac_r and tac_f, p13_r and p13_f, and pJ23104_r and pJ23104_f, respectively. All the plasmids were verified by PCR, restriction digestion, and sequencing (Figure S4).

2.3.3. Construction of Producer−Sensor Plasmids. Two producer−sensor plasmids (pRha_ISP_AcIa_P_c and pRha_ISP_AcIa_P_{tac}) were designed (Figure S8), where the producer component included the isoprene synthase (*ispS*) gene from *Populus alba* under the control of the rhamnose-inducible P_{RhaRS} system, and the sensor component was taken from the constructs pAcIa_P_c and pAcIa_P_{tac}. First, pSA_Rha_rfp was made by amplifying the rhamnose promoter using the primer pair pRha_f and pRha_r with *E. coli* MG1655 gDNA as the template and introducing it in pSA_MP at the *Eco*RI and *Spe*I restriction sites. The nucleotide sequence of the *ispS* gene was synthesized from GenScript Biotech Corporation and amplified using the primer pair IspPA-inf_f and ispS-B1002_r, while the complete *sensor-rfp* sequence was amplified using B1002-cHyb_f and T7Te-sensor_r with pAcIa_pc and pAcIa_tac as the templates. Infusion cloning (3-fragment) was carried out with PCR-amplified *ispS*, with *sensor-rfp* fragments and *Nde*I-*Pst*I digested SA_Rha_RFP to construct the rhamnose-inducible producer−sensor plasmids pRha_ISP_AcIa_P_c and pRha_ISP_AcIa_P_{tac}.

2.4. Construction of the *E. coli* MG1655_KO Strain. CRISPR-based knockout of the *araC* gene was undertaken following the author's recommended protocol.[37] Briefly, plasmid pCAS (Addgene Plasmid 62225) was electroporated into *E. coli* MG1655 cells. Following L-arabinose induction, the pCAS-transformed *E. coli* MG1655 was made electrocompetent. Simultaneously, two guide RNAs (gRNAs) were designed targeting the *araC* gene loci. Platinum SuperFi II PCR Master Mix (Invitrogen) was used to amplify the whole plasmid pTargetF (Addgene Plasmid 62226) with the primer pairs gRNA1_f and gRNA_r and gRNA2_f and gRNA_r, independently, where the designed gRNAs were included as overhangs in the oligonucleotides gRNA1_f and gRNA2_f, respectively. The PCR-amplified pTargetF was digested with *Spe*I followed by ligation and transformation to get pTargetF_g1 and pTargetF_g2 carrying two different gRNAs. Oligonucleotide pairs HA_1_f and HA_1_r, and HA_2_f and HA_2_r were used to amplify the homology arms HA_1 and HA_2, respectively using the *E. coli* MG1655 gDNA as the template. Plasmids pTargetF_g1 and pTargetF_g2 were separately digested with *Sal*I and *Pst*I, followed by 3-fragment infusion cloning with fragments HA_1 and HA_2 and transformation in chemically competent DH5α cells to form plasmids pTargetF_g1_HA and pTargetF_g2_HA, respectively. These plasmids were electroporated separately into pCAS-transformed electrocompetent *E. coli* MG1655 and allowed to grow. Individual colonies were picked and induced with IPTG followed by growth at 37 °C to cure both the plasmids. Colonies were screened by colony PCR and arabinose auxotrophy. The *araC* knockout strain *E. coli* MG1655_KO was verified by Sanger sequencing (Figure S5). All the verified plasmids were transformed into *E. coli* MG1655, *E. coli* MG1655_KO, *E. coli* MG1655 ΔAraC, and *E. coli* MG1655 ΔAraC ΔAraBAD strains to assess the biosensor activity.

For reintroducing the araC into the KO strain, the construct pC_AraC_AcIa_P_{tac} was made. The sensor-*rfp* portion along with the terminators on both sides was PCR-amplified from the template plasmid pRha_ISP_AcIa_P_{tac} using the primer pair RT1_f and RT1_r, followed by digestion with *Nhe*I and *Pst*I. The plasmid pSA_BAD_rfp was digested with restriction enzymes *Xba*I and *Pst*I and ligated with the digested insert to give the construct pC_AraC_AcIa_P_{tac}, which was sequence-verified.

2.5. Biosensor Assay. Individual colonies were picked and grown overnight in M9 minimal medium supplemented with 40 μg L^{-1} chloramphenicol. For the assay, 0.5 mL of the overnight culture was added to 9.5 mL of fresh M9 minimal medium in 20 mL gas-tight serum bottles and grown for 3 h at 30 °C and 180 rpm in a shaking incubator. Samples were induced with arabinose (1 mM) or/and isoprene (varying concentrations) using a Hamilton GASTIGHT syringe, 1800 series 1801RN (volume 10 μL) (Hamilton). Six hours post-

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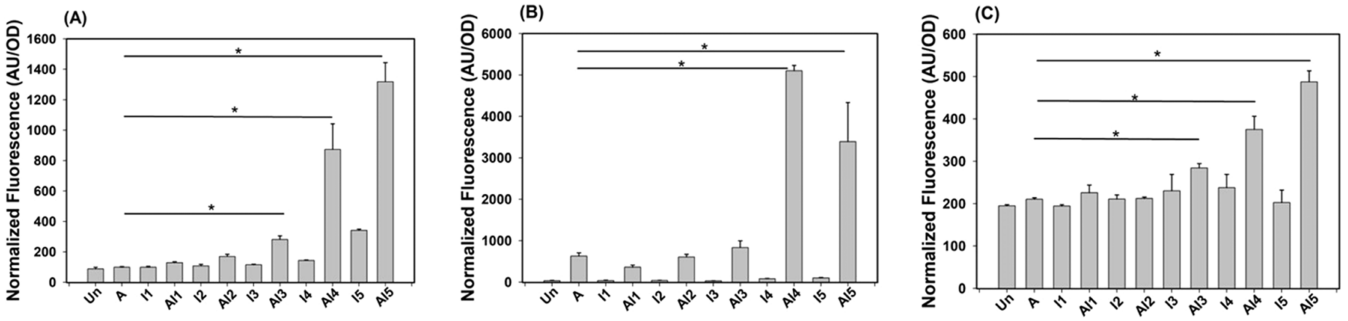

Figure 1. Assessment of biosensor pAcIa_P_c in different growth media using fluorescence-based assays. *E. coli* MG1655 transformed with pAcIa_P_c was cultured in (A) M9 minimal media supplemented with 4 g L^{-1} glucose, (B) tryptone broth, and (c) M9 minimal media supplemented with 4 g L^{-1} glucose and 5 g L^{-1} yeast extract. Cultures were induced with 1 mM arabinose (A) and/or varying concentrations of isoprene (I1 = 1.25; I2 = 2.5; I3 = 5; I4 = 7.5, and I5 = 10 mM). The error bar represents the standard error for triplicates. Asterisks (*) denote a p-value less of than 0.005.

induction, 200 μL of the culture was added to a 96-well black/clear bottom plate (Thermo Scientific), and the RFP fluorescence was recorded with the excitation wavelength set at 585 nm and emission wavelength of 620 nm, and absorbance was measured at 600 nm. Normalized fluorescence and fold change was calculated using the following equations

$$\text{normalized fluorescence} = \frac{\text{fluorescence(sample)} - \text{fluorescence(blank)}}{\text{absorbance(sample)} - \text{absorbance(blank)}} \quad (1)$$

$$\text{fold change} = \frac{\text{normalized fluorescence(induced sample)}}{\text{normalized fluorescence(uninduced sample)}} \quad (2)$$

2.6. Isoprene Estimation. The isoprene concentration in the headspace was measured using a 7820A gas chromatograph (Agilent Technologies) equipped with a FID detector, fitted with a HP-5MS column (30 m × 0.320 mm × 0.25 μm) (Agilent). 100 μl of the headspace gas from the gastight serum bottles in which the cultures were grown was collected using a Hamilton syringe, 1000 series GASTIGHT (1 mL volume) (Hamilton), and injected into the gas chromatograph. The gas chromatography (GC) oven was initially maintained at 30 °C for 3 min and then ramped to 120 °C at a rate of 20 °C/min.[38] The inlet temperature was set to 220 °C, and the detector temperature was set at 180 °C. Isoprene was eluted with a retention time of 2.2 min.

2.7. RNA Extraction and qRT-PCR. RNA extraction and qPCR were performed as described previously.[39] Briefly, 5 mL of culture was collected and the RNA was extracted using PureZOL RNA Isolation Reagent (Bio-Rad) following the manufacturer's recommendations. DNase treatment was undertaken using TURBO DNase (Invitrogen), and samples were purified using the RNeasy Mini Kit (Qiagen). Complementary DNA was synthesized by using the Prime-Script II 1st strand cDNA Synthesis Kit (Takara Bio Inc). Gene-specific oligos (Table S1) were used for qRT-PCR using PowerUp SYBR Green Master Mix (Applied Biosystems) in a StepOne real-time PCR system (Applied Biosystems).

2.8. Protein Extraction and Gel Electrophoresis. 10 ml of the culture, following the fluorescence assay, was harvested and washed with 1× phosphate-buffered saline (PBS). The pellet was resuspended in 1× PBS and divided into three parts—for native polyacrylamide gel electrophoresis (PAGE), for sodium dodecyl sulfate—polyacrylamide gel electrophoresis (SDS-PAGE), and one part for reaction with the crosslinking agent disuccinimidyl suberate (DSS, Thermo Scientific) followed by SDS-PAGE. The cell pellet sample for the native PAGE was resuspend in lysis buffer (including 0.1 mg mL^{-1} lysozyme, 1 mM PMSF, 0.02 mg mL^{-1} DNase, and 5 mM $MgCl_2$) and kept on ice for 30 min. The samples were spun down and mixed with 2× sample buffer and run on a 10% native gel. The cell pellet for SDS-PAGE was boiled with 2× SDS sample buffer and run on 10% SDS-PAGE gel. For the third set, the cell pellet was resuspended in 250 μL of 1× PBS, and the DSS crosslinking agent was added to a final concentration of 1 mM. After incubation at room temperature for 30 min, 1 M Tris was added to stop the reaction. Following 15 min of incubation at room temperature, the samples were spun down, boiled with 2× SDS sample buffer, and separated on a 10% SDS-PAGE gel. Puregene-prestained protein ladder 10−250 kDa (Genetix Biotech Asia Pvt Ltd) was used as the protein marker.

3. RESULTS

3.1. Design of the Isoprene Biosensor. The arabinose-inducible system is one of the most extensively studied and used inducible promoter systems in regulating the gene expression. The AraC dimers bind to O2-I1 half-sites in the absence of L-arabinose to prevent transcription by forming a DNA loop structure. On binding to L-arabinose, the AraC homodimers change the orientation of the DNA-binding domains (DBDs), enabling them to bind to I1-I2 half-sites and facilitating transcription of the downstream gene.[27,28] AraC has been evolved to recognize other molecules such as D-arabinose, mevalonate, ectoine, triacetic acid lactone, etc., in addition to L-arabinose.[24,29−31] Few studies have reported the use of chimeric AraC, where the ligand-binding domain (LBD) is replaced with dimerizing proteins/domains, to switch the ligand specificity of the system.[25,32]

In our preliminary studies, we noted that the AraC-pBAD system showed sensitivity to isoprene, when arabinose was also present. In this study, we attempted to develop a biosensor explicitly for isoprene by designing a chimeric AraC transcription factor. Isoprene monooxygenase (IsoMO) is a 6-protein complex composed of IsoABCDEF. The oxygenase component (Iso ABE) catalyzes the conversion of isoprene to isoprene monoxide, in the isoprene metabolism pathway found in the isoprene-utilizing organism *Rhodococcus sp.* AD45.[35,40] Literature reports suggest that IsoA (isoprene monooxygenase α subunit or propane monooxygenase) forms the core active

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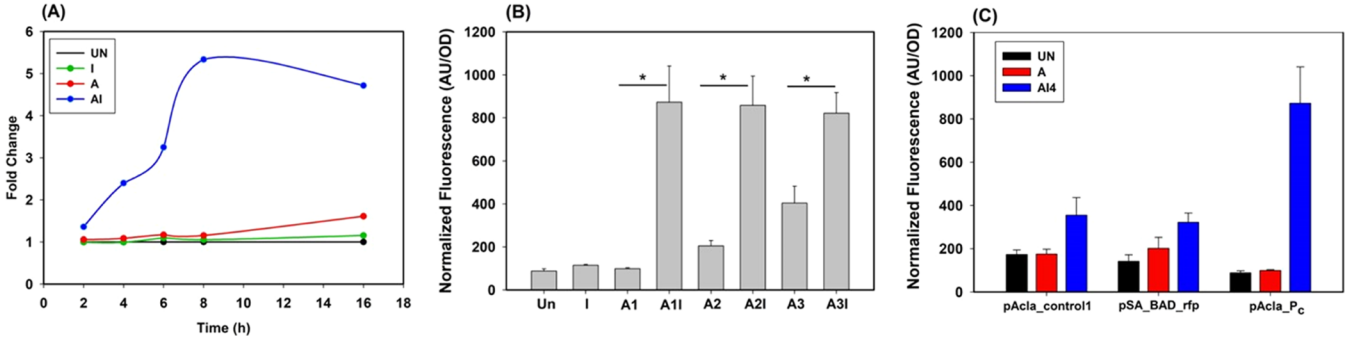

Figure 2. Characterization of biosensor pAcIa_P_c transformed in *E. coli* MG1655 through fluorescence assays. (A) Time course assay of the biosensor. Fluorescence measurements were taken 2, 4, 6, 8, and 16 h post-induction with only isoprene (I, green; 5 mM), only arabinose (A, red; 1 mM), and both isoprene and arabinose (AI, blue). (B) Effect of varying arabinose concentration on biosensor activity. Cultures were induced with 7.5 mM isoprene (I) and/or varying concentrations of arabinose (A_1 = 1, A_2= 5, A_3 = 10 mM). (C) Comparative fluorescence assay for *E. coli* MG1655 transformed with control plasmids pAcIa_control1 and pSA_BAD_rfp with the biosensor pAcIa_P_c induced with only arabinose (red; 1 mM) and both arabinose (1 mM) and isoprene (7.5 mM) (blue). The error bar represents the standard error for triplicates. The asterisk (*) denotes a *p*-value less than 0.005.

site for the binding of isoprene.[34] Hence, we wanted to explore if a nondimerizing protein, like IsoA, can be used for making a functional chimeric AraC, which can bind and specifically respond to isoprene.

For this purpose, a chimeric AraC (henceforth referred to as AcIa) was designed with the DBD (178−291 amino acids) and linker sequence (171−178 amino acids) of *araC* in frame with the complete nucleotide sequence of *isoA* (CAB55825) (sequence in the Supporting Information). This AcIa replaced the AraC gene in the native AraC-pBAD promoter system and was introduced with a *rfp* reporter gene into pSA_MP to form our primary biosensor construct pAcIa_P_c (Figure S1).

3.2. Characterization of the Biosensor pAcIa_P_c. The biosensor plasmid pAcIa_P_c was transformed into *E. coli* MG1655 cells and characterized for its activity by fluorescence-based assays. When transformed cells were induced with only isoprene, no fluorescence was observed 6 h post-induction. However, when cultures were induced with both isoprene and arabinose, a fluorescence readout was obtained. In order to increase the signal-to-noise ratio and minimize the leaky expression of the reporter gene due to arabinose, a lower concentration (1 mM) of arabinose was used in these assays. It was interesting to note that in the presence of only isoprene or arabinose, the system was not significantly induced but was triggered only when both were present.

Fluorescence-based assays were undertaken in different growth media to test the performance and sensitivity of the biosensor to isoprene; the cells were induced with different isoprene concentrations from 1.25 to 10 mM, with and without arabinose (1 mM) (Figure 1). Due to its highly volatile nature, inducing cultures with isoprene lower than 1.25 mM concentration was very difficult and erroneous. When cultured in M9 minimal media with glucose, the fluorescence values observed for cultures induced only with arabinose were much lower, and significant sensitivity to isoprene concentrations above 2.5 mM was observed, with ∼10-fold and ∼15-fold higher fluorescence readouts for 7.5 and 10 mM isoprene concentrations, respectively (Figure 1A). However, when assays were carried out in richer media such as tryptone broth, higher fluorescence was observed in the presence of arabinose (only), resulting in a lower signal-to-noise ratio (Figure 1B). Assays were also carried out in M9 minimal media with the yeast extract; however, much lower sensitivity was observed, although the induction trend was similar (Figure 1C). Hence, all biosensor experiments were conducted in M9 minimal media. Trial experiments of the biosensor in M9 media supplemented with glycerol, instead of glucose, were also undertaken. Amplified fluorescence was observed with the addition of arabinose (without isoprene), while a reduction in fluorescence was noted on induction with isoprene (Figure S2). This was also noted with SA_BAD_rfp and complements the previous literature reports, which suggest a possible absence of catabolite repression in glycerol grown cultures.[27,41−43]

To further characterize the pAcIa_P_c biosensor, a time course experiment was carried out. Cultures were subjected to 5 mM isoprene and 1 mM arabinose, and the fluorescence values were noted at specific time points (2, 4, 6, 8, and 16 h) post-induction (Figure 2A). The highest level of fluorescence was recorded after 8 h and dropped slightly by 16 h, probably due to the consumption of the supplemented arabinose.

Since the fluorescence was noted only in cultures which were also supplemented with arabinose, it was imperative to test the biosensor in varying arabinose concentrations. For this, the cultures were induced with different arabinose concentrations (1, 5, and 10 mM) without or with (7.5 mM) isoprene (Figure 2B). Although higher basal fluorescence was observed in cultures induced with a higher concentration of arabinose, a twofold higher fluorescence was still observed for cultures induced with 10 mM arabinose and 7.5 mM isoprene. It was also interesting to note that the normalized fluorescence of cultures subjected to both arabinose and isoprene was similar, irrespective of the concentration of arabinose added.

In order to understand the working of the biosensor, it was critical to design control plasmid pAcIa_control1, which lacked the *AcIa* gene. Plasmid pSA_BAD_rfp was also assayed to compare and ensure that the AcIa displayed higher sensitivity for isoprene than that natively displayed by AraC. Fluorescence assays with these two constructs (map in Figure S3) were undertaken, and cultures transformed with these plasmids were induced with isoprene (7.5 mM) and arabinose (1 mM) (Figure 2C). Induction with isoprene was observed in both pAcIa_control1 and pSA_BAD_rfp when supplemented with arabinose, albeit to a lower extent than pAcIa_P_c. The fluorescence noted in pAcIa_control1 can be attributed to the copy of *araC* in the wild-type *E. coli* MG1655 genome.

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These experiments complemented our preliminary studies (data not shown), where we observed a minor induction of the native AraC-pBAD system when supplemented with both arabinose and isoprene. These experiments also suggested that both AraC and AcIa transcription factors could trigger the P_{BAD} promoter on induction with arabinose and isoprene, with a more heightened response to isoprene being noted for AcIa.

3.3. Biosensor Variants. In the absence of arabinose, the native pBAD promoter folds to form a DNA loop, with AraC dimers attaching to the O2-I1 half-sites. In order to prevent this autoregulation, two variants of the pAcIa_P_c biosensor were designed, pAcIa_wo2 which lacks the operator O2 half-site only and pAcIa_wo1o2 in which both O1 and O2 sites were absent (Figure 3A). These constructs were introduced into *E. coli* MG1655 and assayed to test the biosensor activity.

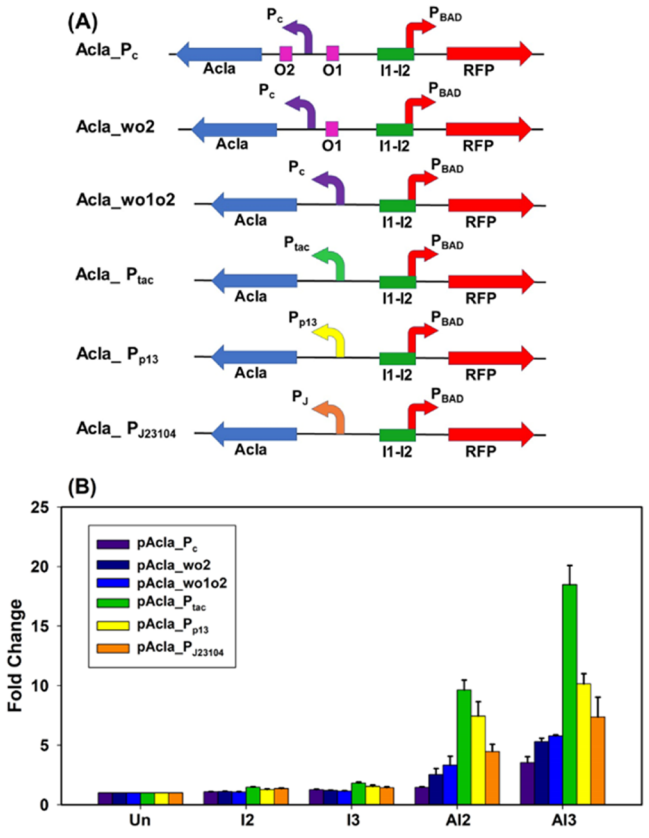

Figure 3. Design and characterization of variant constructs of the isoprene biosensor in *E. coli* MG1655. (A) Schematic representation of the isoprene biosensor components. AcIa_P_c includes chimeric AraC(DBD)-IsoA represented as AcIa (in blue), O1 and O2 (in pink), I1-I2 half-sites (in green), native promoters of the arabinose-inducible system P_{AraC} (purple) and P_{BAD} (red), and reporter gene *rfp* (in red). Other variant constructs include AcIa_wo2 which lacks the O2 half site; AcIa_wo1o2 which lacks both O1 and O2 sequences; AcIa_P_{tac} which has promoter P_{tac} instead of P_c; AcIa_P_{p13} which has promoter P_{p13} instead of P_c; and AcIa_P_{J23104} which has promoter P_{J23104} instead of P_c. (B) Fluorescence-based assay to characterize the biosensor variants AcIa_wo2 (dark blue), AcIa_wo1o2 (blue), AcIa_P_{tac} (green), AcIa_P_{p13} (yellow), and AcIa_P_{J23104} (orange). Cultures were induced with only isoprene (I2 = 2.5 and I3 = 5 mM) and with arabinose (1 mM) and isoprene (AI2, AI3). Fluorescence (ex = 585 nm, em = 620 nm) and absorbance (600 nm) measurements were taken 6 h post-induction. Normalized fluorescence was calculated using eq 1 in Section 2. Error bars represent the standard deviation for biological triplicates.

RFP fluorescence was measured 6 h post-induction with two concentrations of isoprene (2.5 and 5 mM) with and without arabinose (1 mM). Both pAcIa_wo2 and pAcIa_wo1o2 showed sensitivity to even 2.5 mM isoprene, with ∼2- and ∼3-fold higher fluorescence, respectively. However, the variants without the operator sequences still required the addition of arabinose to trigger the regulation by isoprene.

To further increase the sensitivity and performance of the biosensor, the native promoter P_c (or P_{AraC}) which controls the expression of *AcIa* in our biosensor construct was replaced. The promoter P_c was substituted by the promoters P_{tac}, P_{p13},[13] and P_{J23104} (BBa_J23104) to make the constructs pAcIa_P_{tac}, pAcIa_P_{p13}, and pAcIa_P_{J23104}, respectively (Figures 3A and S4). All the constructs with heterologous promoters showed increased sensitivities and higher normalized fluorescence values with isoprene (in the presence of arabinose) as compared to that observed with pAcIa_P_c in *E. coli* MG1655. The sensor pAcIa_P_{tac} showed the highest sensitivity, followed by pAcIa_P_{p13} with the least shown by pAcIa_P_{J23104} (Figure 3B).

3.4. Characterization of pAcIa_P_{tac}. It was interesting to note that the pAcIa_P_{tac} gave a sixfold higher induction with 5 mM isoprene as compared to pAcIa_P_c. Hence, it was imperative to further characterize the pAcIa_P_{tac} sensor construct for its sensitivity and specificity. *E. coli* MG1655 cells carrying the pAcIa_P_{tac} sensor were induced with varying isoprene concentrations (1.25−10 mM) with and without the supplementation of arabinose (1 mM) (Figure 4A). Higher normalized fluorescence values were observed only in cultures that were induced with both isoprene and arabinose. A nearly proportional increase in the fluorescence output was observed with increasing isoprene concentrations, with the signal saturating at 7.5 mM isoprene. When induced with 7.5 mM isoprene (and arabinose), the cells displayed a 64-fold higher fluorescence which was ∼7 times that observed with pAcIa_P_c for the same induction. It was also critical to note that the pAcIa_P_{tac} sensor construct showed improved sensitivity to lower isoprene concentrations, with a 2.5-fold induction even for 1.25 mM isoprene.

Another crucial characteristic of a good biosensor is its specificity to the ligand of interest. Isoprene is oxidized to isoprene monoxide by the isoprene monooxygenase complex (IsoABCDEF).[34] Our biosensor contains IsoA; hence, it was necessary to test the sensitivity of the sensor to isoprene monoxide as well. The earlier reported biosensor for isoprene was a toluene biosensor, which was also triggered by isoprene, however to a lesser extent.[33] Hence, it was important to test if toluene could also trigger our biosensor. *E. coli* MG1655 transformed with the pAcIa_P_{tac} sensor was assayed for the biosensor specificity by inducing cultures with 2.5 mM isoprene monoxide, 5 mM isoprene monoxide, and 5 mM toluene with and without arabinose supplementation (1 mM). No significant induction was observed with isoprene monoxide and toluene (Figure 4B). The higher normalized fluorescence values in toluene-induced cultures was due to the lower absorbance values recorded, possibly due to toxicity caused by toluene.

3.5. Biosensor Activity in *E. coli* MG1655 AraC Knockout Strains. To further investigate the working of the biosensor, we introduced the pAcIa_P_{tac} in *E. coli* MG1655 strains, in which the *araC* gene was knocked out. For this purpose, we designed and constructed a knockout strain using the CRISPR-Cas technique.[37] Two guide RNAs were designed

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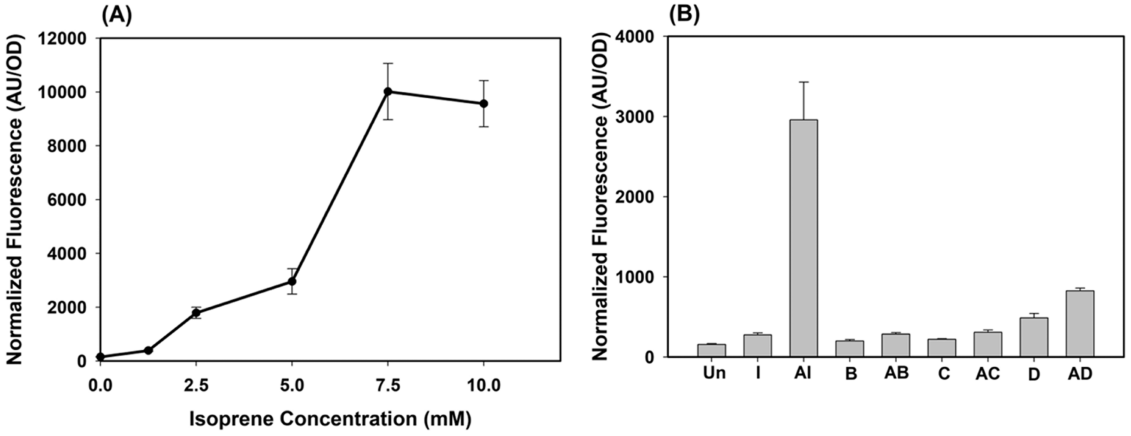

Figure 4. Characterization of the AcIa_P_{tac} sensor in *E. coli* MG1655. (A) Dynamic range of the AcIa_P_{tac} biosensor in *E. coli* MG1655. Cultures were induced with arabinose (1 mM) and isoprene (1.25−10 mM). Fluorescence and absorbance were measured at 6 h post-induction. (B) Specificity of the AcIa_P_{tac} biosensor was tested by inducing the cultures with 5 mM isoprene (I; AI (with 1 mM arabinose)), 2.5 mM isoprene monoxide (2-methyl-2-vinyloxirane) (B, AB (with 1 mM arabinose)), 5 mM isoprene monoxide (C, AC (with 1 mM arabinose)) and 5 mM toluene (D, AD (with 1 mM arabinose)). Error bars represent the standard deviation for biological triplicates.

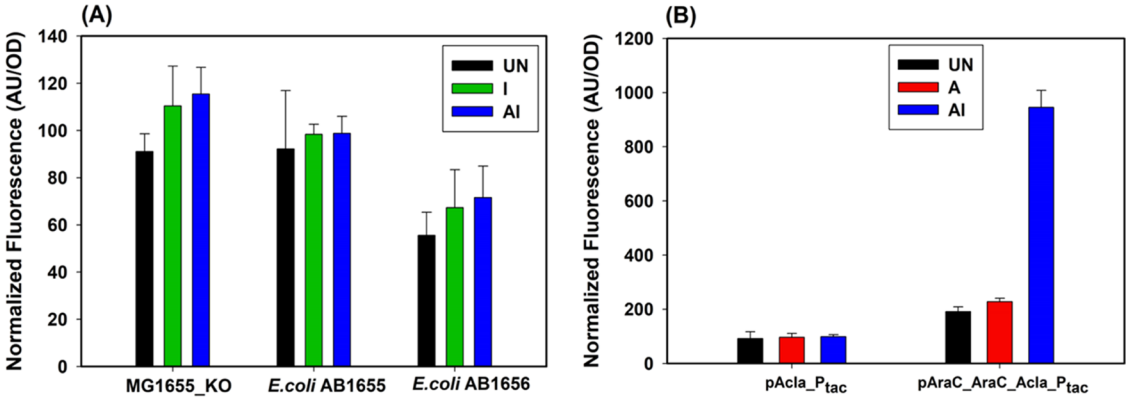

Figure 5. Biosensor activity in AraC knockout strains. (A) Normalized fluorescence for biosensor pAcIa_P_{tac} transformed into AraC knockout strains *E. coli* MG1655_KO, *E. coli* AB1655, and *E. coli* AB1656, induced with 7.5 mM isoprene only (green) and both 7.5 mM isoprene and 1 mM arabinose (blue). (B) Normalized fluorescence for biosensor pAcIa_P_{tac} and construct P_C_AraC_AcIa_P_{tac} in AraC knockout strain *E. coli* MG1655_KO induced with 1 mM arabinose (red) and with both arabinose (1 mM) and 7.5 mM isoprene (blue). Error bars represent standard deviation for biological triplicates.

targeting the *araC* loci and used to construct the plasmids pTargetF_g1 and pTargetF_g2. Knockout colonies showing auxotrophy for arabinose were obtained only with gRNA_1 (seq: CAGAATCACTGCCAAAATCG). The colony was isolated, sequence-verified, and referred to as *E. coli* MG1655_KO (Figure S5). We also got MG1655 ΔaraC (*E. coli* AB1655) and MG1655 ΔaraC ΔaraBAD (*E. coli* AB1656) strains from Prof. Mustafa Khammash (ETH Zurich) as a kind gift. The plasmid pAcIa_P_{tac} was transformed into all three knockout strains, and the fluorescence assays were repeated as earlier. The fluorescence values were very low with no significant increase in the induced condition even with both isoprene and arabinose (Figure 5A).

To test the essentiality of AraC for biosensor functionality, the plasmid P_C_AraC_AcIa_P_{tac} carrying AraC and the biosensor pAcIa_P_{tac} was transformed into the knockout strain *E. coli* MG1655_KO (Figure 5B). When induced with arabinose and isoprene, a fourfold increase in the fluorescence was observed. This experiment validated that AraC is indispensable for the functioning of our biosensor.

To understand the possible mechanism of functioning of the pAcIa_P_{tac} sensor, we examined the transcript abundance levels of the genes *araC*, *AcIa*, and *rfp* (Figure S6). To distinguish between the transcript abundance of *araC* and *AcIa*, the qPCR primers for *araC* were designed for the LBD region and those for *AcIa* were targeted to *isoA*. As the expression of *AcIa* is driven by the constitutive promoter P_{tac}, the relative mRNA abundance of *AcIa* was expected to be similar irrespective of the addition of inducers. However, it was the highest for cultures induced with both isoprene and arabinose, but the difference observed was not statistically significant. Nonetheless, as expected, the transcript abundance *araC* was indeed significantly higher for the cultures supplemented with arabinose (A and AI conditions). The *rfp* gene expression levels complemented the *AcIa* expression profile, with the maximum abundance in the AI induction set and slightly amplified levels in cultures supplemented with only arabinose. The arabinose dependence for triggering the biosensor response to isoprene, and the transcript abundance levels suggest the possible essentiality and interaction of AraC, AcIa, and isoprene for inducing the fluorescence gene expression.

From our experiments, we infer that AraC is essential for the biosensor functioning; however, greater sensitivity to isoprene

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concentrations is observed when the expression of *AcIa* is enhanced. To further investigate if a potential dimerization or oligomerization of AraC and AcIa in the presence of isoprene was responsible for switching on the system, we undertook protein gel analysis. Total cell lysate of pAcIa_P_{tac} along with controls pAcIa_control1 and pSA_BAD_rfp (for induced and uninduced condition) were analyzed on the native protein gel and SDS-PAGE gels (Figure S7). Additionally, the cell pellet was treated with the crosslinking agent DSS, expecting it to stabilize the potential interaction between AraC and AcIa, enabling the detection of a complex by SDS-PAGE. Although the distinct band corresponding to RFP was observed both in native and SDS- PAGE gel, the DSS did not provide the expected insights into the possible mechanism. More in-depth focused exploration of the protein interactions by using anti-AraC antibodies, size exclusion chromatography or in vitro assay of purified proteins is required to gain clear insights into the possible mechanism of functioning of this chimeric biosensor.

3.6. Producer−Sensor Constructs. The fluorescence assays with the biosensors were done at a minimum concentration of 1.25 mM isoprene as measuring volumes lower than this using a gas-tight syringe were not possible. Induction with saturated solution led to lot of variability in the amount of isoprene added between replicates due to the lower solubility of isoprene and its volatile nature. Therefore, to further test the sensitivity of the biosensors as well as to demonstrate the application of our biosensor for real-time monitoring and use in genetic circuits, producer−sensor constructs were constructed. *E. coli* strains use the native methyl-erythritol phosphate (MEP) pathway to produce isoprenoids.[6,9] By the heterologous introduction of a single enzyme (isoprene synthase) catalyzing the conversion of DMAPP (dimethylallyl diphosphate) to isoprene, *E. coli* cells can be made to produce isoprene via the MEP pathway.[6,38,44] Hence, the producer−sensor construct included the producer component composed of the *isoprene synthase* gene (*ispS*) from *P. alba* under the control of the rhamnose-inducible promoter system (P_{RhaRS} promoter system) and the sensor component (biosensors pAcIa_P_c or pAcIa_P_{tac}), which was separated by a terminator T_{b1002} (BBa_B1002_terminator) (Figure S8).

In the construct pRha_ISP_AcIa_P_c (referred to as RH), the expression of *AcIa* was regulated by the promoter P_{araC} (or P_c), while in pRha_ISP_AcIa_P_{tac} (referred to as RT), *AcIa* was under the control of promoter P_{tac}. Both RH and RT plasmids were transformed into *E. coli* MG1655. Cells were grown in M9 minimal media with glucose and induced with rhamnose (10 mM) and/or arabinose (1 mM). In a previous study, glycerol was added to enhance isoprene production[33] by cells. However, as suggested in the literature reports for the araC-pBAD system and as also noted in our biosensor assay (Figure S2), an amplified induction is observed for cultures grown in glycerol possibly due to the absence of catabolite repression.[27,41−43] Hence, producer−sensor strains were grown in media supplemented only with glucose. Fluorescence measurements and isoprene estimation were undertaken 24, 48, and 96 h post-induction. Isoprene was detected in the cultures induced with rhamnose after 48 h of induction. Isoprene concentrations of 0.596 and 1.047 μM were noted in cultures 48 h and 96 h, respectively, post-induction with rhamnose, which complements the level reported in literature.[45] Cells transformed with RH did not show any significant increase in fluorescence in both 48 h and 96 h samples (data not shown); while 96 h post-induction, RT showed ∼2-fold higher fluorescence in cultures induced with rhamnose and arabinose compared to the background noise produced by RT cultures induced only with arabinose (Figure 6). However, based on the biosensor response, the estimated

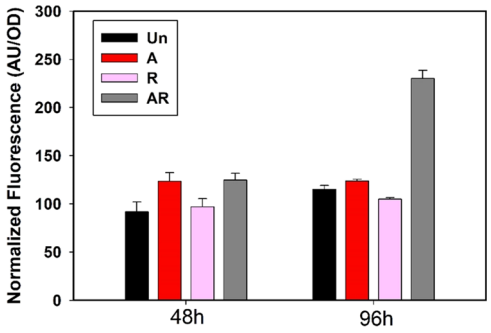

Figure 6. Normalized fluorescence for producer−sensor strain. Construct pRha_ISP_AcIa_P_{tac} (RT) was transformed into *E. coli* MG1655 and induced with only arabinose (1 mM; A) only rhamnose (10 mM; R), or both rhamnose and arabinose (AR). Fluorescence measurements and isoprene estimation were done 48 and 96 h post-induction. Error bars represent the standard deviation for biological triplicates.

isoprene concentration in RT cultures (96 h) was 0.331 mM, which was about 300 times higher than that measured. The discrepancy in the estimated and measured values could be partly attributed to the difference in intracellular and extracellular concentrations of isoprene. The intracellular concentration in the producer strain is expected to be much higher than the extracellular headspace concentration. Additionally, due to the volatile nature of isoprene, making accurate dilutions of very low concentrations for the GC standard curve was not possible, possibly causing a slight bias in the measured values.

4. DISCUSSION

The AraC-pBAD system has been adapted natively for sensing different small molecules or engineered to develop chimeric transcription factors for responding to metabolites or even light. In our preliminary studies, we noted induction of the native AraC-pBAD system by isoprene (when arabinose was also added) albeit to a lower extent. Although the AraC homodimer triggered the reporter gene expression at higher isoprene concentrations, a biosensor which displays a more sensitive and specific response to isoprene is preferred. In this study, we engineered the AraC-pBAD system to increase its specificity for isoprene by replacing the ligand-binding domain of AraC with IsoA. Contrary to earlier reports on chimeric AraC that used proteins/domains which are known to dimerize, we sought to develop chimeric AraC with IsoA, which is a component of a multiprotein complex.

In our study, we noticed the lowest background signal and highest fold change when the assay was carried out in minimal medium with glucose. In our assay, the cultures are grown in glucose-supplemented minimal medium for 2 h followed by induction with arabinose and isoprene. Our time course experiments showed the increase in response with progression of time, possibly due to the shift from repressing to inducing conditions with the consumption of glucose in the medium,[46] which also possibly contributed to a higher induction-to-

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repression ratio. Additionally, it has been reported that the induction/repression ratio is lower in rich media as compared to minimal media,[46] which validates our observations.

With the *AcIa* chimeric transcription factor, a better response to isoprene levels was noted, however, only in the presence of arabinose. By swapping the native P_{araC} with the P_{tac} promoter and removing the operator sequences, the sensitivity of the system to isoprene was greatly enhanced, but it still required the supplementation of arabinose. The loss of biosensor activity in the knockout strains and restoration of functionality on reintroduction of AraC emphasized that AraC is indispensable for the functioning of our biosensor. These observations suggested two probable mechanisms for the biosensor working; either the native AraC homodimer alone was responsible for the response observed (irrespective of the biosensor constructs) or some interaction between AraC, AcIa, and isoprene switched on the fluorescent gene expression of the biosensor.

The difference in sensitivity and fluorescence response observed for pAcIa_P_c and its variants (especially the significantly different response noted with different promoters (P_{tac}, P_{p13}, and P_{J23104})) advocates that the level of expression of AcIa directly correlates to the response of the biosensor. Additionally, if the biosensor response was indeed solely driven by the AraC homodimer, the fluorescence readout for pSA_BAD_rfp should have been much higher than that of pAcIa_control1 in our control experiment, as the AraC levels in the former would be much higher (due to the higher plasmid copy number) than the latter (one copy of AraC in the genome). These findings suggest that AraC is essential for switching on the biosensor, while the level of AcIa is directly linked to the sensitivity of the biosensor to varying isoprene concentrations.

We tried to briefly investigate the possible interaction between AraC and AcIa using protein gels and crosslinking agents. However, conclusive insights and clarity on the interactions could not be established. More specifically, focused experimental studies along with thorough structural analysis of the interaction (both independent and combined) of AraC and AcIa with isoprene is essential for complete understanding of the role of AraC and AcIa in the observed biosensor response. Insights obtained from such studies will be crucial for optimizing the biosensor sensitivity and increasing adaptability to a wider host range not limited to *E. coli*. Despite the certain limitations of the developed biosensor, this study provides the first step toward the possibility of developing such biosensors for a wide range of metabolites.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c01164.

List of oligonucleotides used in this study with their sequences; nucleotide sequence of acIa; plasmid map of our primary biosensor construct pAcIa_P_c; fluorescence-based assay of the biosensor construct pAcIa_P_c transformed in *E. coli* MG1655, cultured in M9 minimal media supplemented with 0.25% glycerol; plasmid map of our control biosensor constructs pAcIa_control1 and pSA_BAD_rfp; multiple sequence alignment of the sequencing results for the promoter region of the constructs AcIa_P_c, AcIa_P_{tac}, AcIa_P_{p13}, and AcIa_P_{J23104} against the template plasmid pAcIa_ P_c; sequencing results for *E. coli* MG1655_KO generated in this study mapped to the AraC-AraBAD locus of the wild-type MG1655 strain; relative mRNA levels of genes; protein gel electrophoresis of pAcIa_P_{tac}, pAcIa_control1, and pSA_BAD_rfp; plasmid maps for the producer−sensor strains (PDF)

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Notes

The authors declare no competing financial interest.

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