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Homogentisic Acid-Based Whole-Cell Biosensor for Detection of Alkaptonuria Disease

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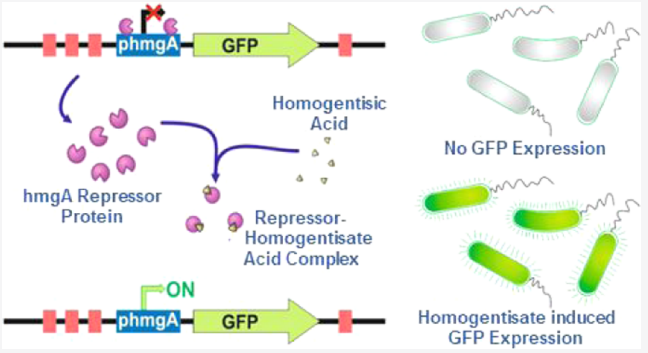
ABSTRACT: Clinicians require simple quantitative tools for the detection of homogentisic acid in alkaptonuria patients, a rare inherited disorder of amino acid metabolism. In this study, we report a whole-cell biosensor for homogentisic acid to detect alkaptonuria disease through the expression of green fluorescence protein. The assay system utilizes a promoter sequence (hmgA) isolated from the *Pseudomonas aeruginosa* genome. To increase the sensitivity, the sensor module harboring *phmgA::GFP* was further transformed into various transposon mutants debilitated in steps involved in the metabolism of phenylalanine and tyrosine via homogentisic acid as a central intermediate. The proposed biosensor was further checked for analytical features such as sensitivity, selectivity, linearity, and precision for the quantification of homogentisic acid in spiked urine samples. The limit of detection for the developed biosensor was calculated to be 3.9 μM, which is comparable to that of the various analytical techniques currently in use. The sensor construct showed no interference from all of the amino acids and its homolog molecules. The accuracy and precision of the proposed biosensor were validated using high-performance liquid chromatography (HPLC) with satisfactory results.



INTRODUCTION

An enhanced level of homogentisic acid in the body is an important biomarker for the pathology of alkaptonuria. Alkaptonuria is a rare autosomal recessive genetic disorder caused by a deficiency of the enzyme homogentisate 1,2-dioxygenase.[1] This enzyme deficiency results in an increased level of homogentisic acid, a product of tyrosine (Tyr) and phenylalanine (Phe) metabolism.[2] Patients with alkaptonuria have a higher accumulation of homogentisic acid in the entire circulation. Urine from alkaptonuria patients contains excessive amount of homogentisic acid at a concentration ranging from 10.81 to 20.84 mM, whereas the urinary homogentisic concentration of healthy individuals is below 72.9 μM.[3] Alkaptonuria disease can be diagnosed from childhood by observing black urine; however, clinical manifestations appear by the third decade of life.[4] Oxidation of homogentisic acid leads to the formation of a pigment polymer that gets deposited in the cartilage, leading to arthritis, mostly in the hip and knee regions.[5] There is currently no cure for alkaptonuria.[6] Phe- and Tyr-restricted diets and ascorbic acid supplements are given to patients of alkaptonuria but with little effect on the disease outcome.[7−10] Only recently, nitisinone (an inhibitor of the enzyme 4-hydroxyphenylpyruvate dioxygenase) has been recommended by the European Medicines Agency (EMA) to be included in the treatment of alkaptonuria.[11] Thus, continuous monitoring of homogentisic acid can help maintain stringent dietary intake of phenylalanine, thereby preventing adverse physical complications. Detection and measurement of homogentisic acid in the urine confirm the diagnosis of alkaptonuria. Analytical techniques like enzymatic spectrophotometry, gas chromatography, and ultra-high-performance liquid chromatography (HPLC) are the most commonly used methods for rapid and sensitive determination of homogentisic acid concentration.[4,12−18] However, these techniques require expensive, specialized instruments with trained researchers, which lead to relatively high analytical cost.[19,33,34]

The use of whole cells as biosensors for in vitro diagnostics offers several advantages such as low cost of production, the ability for point-of-care device development, and self-replicating ability. Biosensors based on bacterial whole cells have been developed to detect metabolites, toxic chemicals, explosives, heavy metals (such as mercury and arsenic), hydroxylated polychlorinated biphenyls in serum, and nitrogen oxides in both serum and urine samples.[19−23] As compared to antibody- or enzyme-based biosensors, whole-cell biosensors

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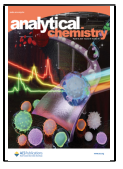

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can report about the target transformations in living systems and thus can report the physiologically relevant data about the analyte. In this study, we report the use of a novel regulatory element isolated from the *P. aeruginosa* (PAO1) genome for direct determination of homogentisic acid concentration spiked in the urine samples. The inability of *Escherichia coli* to mineralize Phe and Tyr led us to scour for metabolic pathways with homogentisic acid as a central intermediate in other microorganisms. We found that *Pseudomonas aeruginosa* metabolizes Phe and Tyr through a peripheral pathway involving hydroxylation of both amino acids by phenylalanine hydroxylase (PhhAB), conversion of Tyr into 4-hydroxyphenylpyruvate by aromatic amino acid aminotransferase (PhhC), and formation of homogentisate by 4-hydroxyphenylpyruvate dioxygenase (Hpd) as the central intermediate (Figure S1).[24,25] Homogentisate is then catabolized by a central catabolic pathway that involves three enzymes, homogentisate dioxygenase (HmgA), fumarylacetoacetate hydrolase (HmgB), and maleylacetoacetate isomerase (HmgC), finally yielding fumarate and acetoacetate.[24]

We utilized this signaling pathway to construct a biosensor for the detection of alkaptonuria by transcriptionally fusing the upstream genetic regulatory promoter region of *hmgA* to the green fluorescence protein (GFP) gene in *P. aeruginosa* chassis. The regulation of *hmgA* gene occurs through the transcriptional regulator (HmgR), which binds directly to its promoter sequence and represses *hmgA* gene expression.[24] Homogentisic acid acts as an inducer for the HmgR regulator, which, in turn, prevents it from binding to promoter sequences of *hmgA*, thereby allowing the expression of *hmgA* gene. We further used a chemical genetics approach to cherry-pick relevant transposon mutants involved in aromatic amino acid degradation to improve the sensitivity of homogentisic acid detection. Of the transposon mutants involved in the degradation of Phe and Tyr, the strain Tn::hmgA carrying the *phmgA::GFP* biosensor construct showed improved fluorescence response for the detection of homogentisic acid. The biosensor construct was checked for analytical features such as sensitivity, linearity, selectivity, and precision for quantitative detection of homogentisic acid, leading to the possible clinical utility of the proposed biosensor for detection of alkaptonuria. The current strategy successfully meets the parameters required to develop a whole-cell biosensor. Besides, this is the first report of a whole-cell biosensor for direct determination of homogentisic acid and demonstrates comparable sensitivity with extant analytical methods in use.

EXPERIMENTAL SECTION

Bacterial Strains, Plasmid, and Chemicals. *P. aeruginosa* PAO1, MPAO1, and various MPAO1 transposon mutants were used as a host for the plasmid-borne transcriptional fusion of the *hmgA* promoter sequence with green fluorescence protein (GFP). Overnight grown cells were inoculated in 3 mL of Luria broth (LB) with 50 μg/mL neomycin under shaking conditions at 180 rpm and 37 °C. Plasmid pPROBE-NT was used as a base vector for the construction of the homogentisic acid biosensor. All amino acids used in this study were procured from HiMedia Laboratories, India. Neomycin and homogentisic acid used in this study were purchased from Sigma Aldrich. Primers used in this study were procured from Sigma Aldrich, India. MPAO1 mutant strains were procured from UW Genome Sciences, Seattle (Table S3). Restriction enzymes used in this study were procured from Thermo Fisher, and New England Biolabs.

Construction of the Biosensing Module. The upstream region of the genetic locus coding for homogentisate dioxygenase gene *hmgA* was introduced into the forward primer (5′-ATTGAAGCTTAAGCAGGTCTCGTTGAG-CAC-3′) that together with the reverse primer (5′-ATTAGAATTCCGCTGGCTGCGTTATTTTTA-3′) was used to amplify the promoter sequence of the *hmgA* gene from the genomic DNA of *P. aeruginosa* (PAO1). Additionally, unique restriction sites (*Eco*R1 and *Hind*III) were introduced in the primers for upstream and downstream regions of the amplified fragment. The resulting PCR product and the vector pPROBE-NT were digested with *Eco*R1 and *Hind*III restriction enzymes, gel purified, and ligated using T4 DNA ligase. The resulting plasmid was transformed into host chassis (PAO1, MPAO1, and transposon mutants) as per the requirement (Figure S2).

Measurement of Fluorescence Intensity. Biosensor strains were grown overnight in LB at 37 °C. The primary culture was then subcultured (1% inoculum) and incubated at 37 °C until OD_{600} reached 0.3. Then, 2% of the culture aliquots were transferred into the assay solution (100 μL) containing varying concentrations of homogentisic acid, 0.3 μM thiamine hydrochloride, 50 μg/mL neomycin, and a mixture of amino acids in 5× minimal medium.[19] Furthermore, 100 μL of the diluted cell culture was then added to a clear-bottom 96-well microtiter plate (Corning 96-well opaque black plate, USA). The plate was incubated at 37 °C for 12 h, during which fluorescence (485 nm excitation/535 nm emission) and absorbance (600 nm) were measured at 30 min intervals using a microplate reader (Synergy H1 multimode plate reader, BioTek). The standard curve was generated using different concentrations of homogentisic acid in the assay solution and plotted against the fluorescence signal. The assay solution was devoid of Phe and Tyr when homogentisic acid was used as a test sample or a standard sample as it is a byproduct of aromatic amino acid catabolism in *P. aeruginosa*.

Screening of MPAO1 Transposon Mutants. In silico analysis of homogentisic acid pathways and the selection of transposon mutants (that can help in increasing the sensitivity of the biosensor) were done on the Pseudomonas Genome DB database[26] and KEGG pathway.[27] The selected transposon mutants were then transformed with the *phmgA::GFP*-based promoter probe vector. The sensitivity of the selected transposon mutants was compared with that of wild-type strain MPAO1.

Fluorescence Microscopy and FACS Analysis. To visualize the bacterial cells expressing the GFP protein in the presence of homogentisic acid using fluorescence microscopy, cells were washed with 1× PBS thrice. Cells were then mounted on the top of the agar pad over a glass slide and were covered with a coverslip for the visualization of cells using fluorescence and differential interference contrast (DIC) microscopy techniques. Cells were then observed under a 100× immersion oil lens of a fluorescence microscope. For FACS experiments, the set of cells analyzed for microscopy was further subjected to FACS analysis (BD FACSVerse flow cytometer; BD Biosciences, USA). For each sample, 10 000 events were recorded. The photomultiplier tube (PMT) voltage settings used were as follows: forward scatter (FSC), 318.8 V; side scatter (SSC), 435.5 V; and fluorescein isothiocyanate (FITC), 261.5 V. Sphero rainbow calibration

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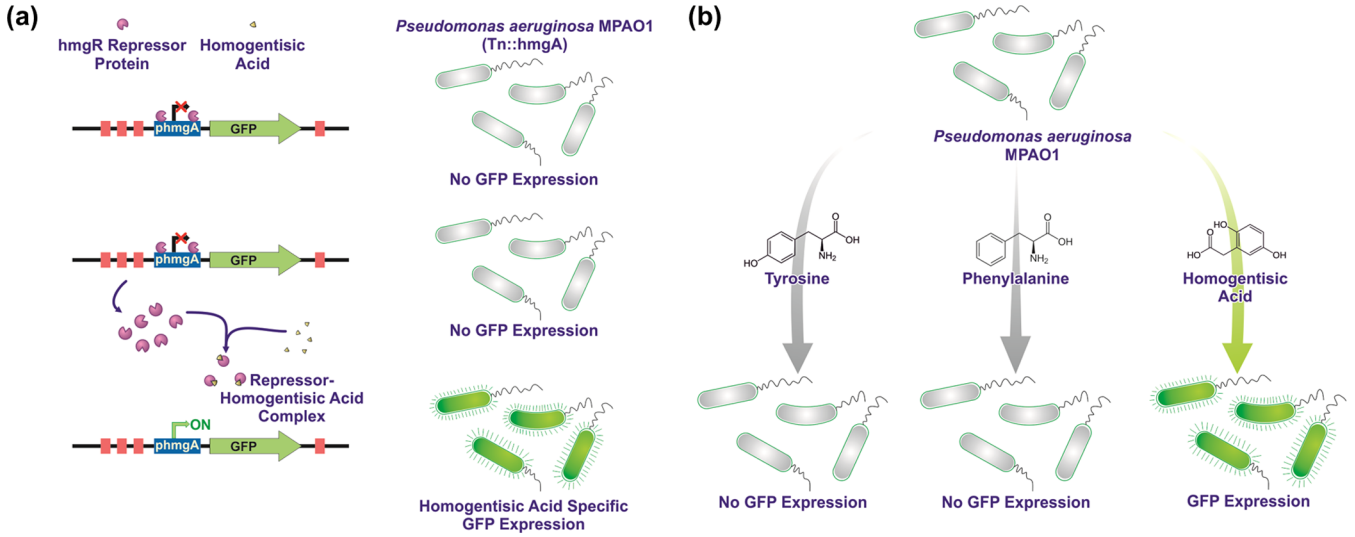

Figure 1. (a) Schematic illustration of a whole-cell-based strategy for the detection of homogentisic acid in alkaptonuria patients. (b) Schematic representation of the selectivity of the proposed biosensor.

particles (BD Biosciences, USA) were used for instrument calibration.

Evaluation of Cross-Reactivity and Linearity. To verify the specificity of the *phmgA::GFP*-based reporter system for homogentisic acid, we examined the fluorescence response of the proposed biosensor with 20 amino acids at a concentration of 160 μM. To assess the linearity of the proposed biosensor, various concentrations of homogentisic acid (2, 5, 20, 50, 80, and 100 μM) were tested in an assay containing 5× minimal medium. Fluorescence intensity was measured every 30 min for 12 h at 37 °C as described above.

Precision for Quantitative Detection of Homogentisic Acid and Diagnosis of Alkaptonuria. The precision of fluorescence measurement was determined by calculating the residuals of the calibrated curves to homogentisic acid. Detection precisions to quantify homogentisic acid in a urine specimen were assessed by the *phmgA::GFP*-based whole-cell biosensor. Urine samples were collected from healthy volunteers and stored at −20 °C before use. The diagnosis of alkaptonuria was performed by adding 2 μL of urine (spiked with different concentrations of homogentisic acid) to 98 μL of 5× minimal media containing an assay solution in a clear-bottom 96-well black plate for cell-based quantification of homogentisic acid. The accuracy of the biosensor was determined by comparing the results to conventional HPLC measurements. The precision and reproducibility of the assay for the diagnosis of alkaptonuria were determined using the coefficient of variation [CV (%) = SD/average × 100] and the recovery rate [recovery (%) = measured value/actual value × 100], resulting from the assay.

Data Analysis. All data were plotted, and statistical analysis was conducted with GraphPad Prism software (GraphPad, California) unless otherwise stated. The lower limit of detection was calculated using the formula described previously[28] from the limit-of-background (LOB) value ($\text{mean}_{(\text{blank})} + 1.645 \times \text{SD}_{(\text{blank})}$) using the equation LLOD = LOB + 1.645 × $\text{SD}_{(\text{lower concentration sample})}$. A linear fitting equation ($y = mx + c$) was fitted for the biosensors, which can be used to predict alkaptonuria. For fold-change data, values obtained with inducer homogentisic acid were divided by values obtained without inducers.[29]

$$\text{fold change}_{(\text{inducer})} = \text{GFP/OD}_{(\text{inducer})}/\text{GFP/OD}_{(\text{inducer=0})}$$

Statistical Analysis. Statistical calculations were performed by analyzing the differences between the treatment and control arms using one-way and two-way analyses of variance (ANOVA) by comparing the mean of each column with the mean of the control column. P values <0.05 were considered significant.

RESULTS AND DISCUSSION

Construction and Characterization of a Genetic Regulatory Element-Based Biosensor for the Detection of Homogentisic Acid. To investigate the possibility for the detection of homogentisic acid leading to the diagnosis of alkaptonuria patients through quantification of green fluorescence protein, the promoter sequence of the *hmgA* gene was transcriptionally fused to the GFP-based promoter probe vector pPROBE-NT (Figure 1a). Further, to check the sensitivity of the biosensor, we transformed the biosensor construct (*phmgA::GFP*) into *P. aeruginosa* PAO1 and MPAO1 strains that were chosen as the host chassis for evaluating the sensitivity of the biosensor module. No increase in the fluorescence intensity (fold change) was observed for our biosensor construct when 0.4% glucose was used as a carbon source in 5× minimal medium, leading to further optimization of the growth conditions (Figure S3). Cells were grown with homogentisic acid in 5× minimal medium supplemented with a mixture of amino acids as described above. In the presence of homogentisic acid (160 μM), the use of the MPAO1 strain as a biosensor chassis displayed better fluorescence response as compared to the PAO1 strain (Figure 3). Besides, the PAO1 strain also showed interference in fluorescence response in the presence of some amino acids, allowing us to prefer the MPAO1 strain as biosensor chassis for further optimization of the biosensor (Figure 3). Genomic and phenotypic variations due to macroevolution could contribute to the differences in the response of these related.[30,31] A recent study has reported that the MPAO1 and PAO1 strains of *P. aeruginosa* show genomic distinctiveness in more than 200 genes. Interestingly, one amino acid permease gene is also encoded uniquely in the MPAO1 genome.[32] The presence of this permease gene could

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Anal. Chem. 2021, 93, 4521−4527

account for transport and thus better response to homogentisic acid by MPAO1 biosensor chassis.

Screening of Transposon Mutants for Better Sensitivity. Based on the in silico analysis of the metabolic pathways for Tyr and Phe, we screened the transposon mutant that could lead to a better biosensor in terms of sensitivity and fold fluorescence response. Thus, the *hmgA*-based biosensing construct was transformed into the selected transposon mutant strains of MPAO1 as host chassis. All of the strains were grown in the assay solution composed of 5× minimal medium as described above with various concentrations of homogentisic acid. Figure 2 shows the dose-dependent response of the

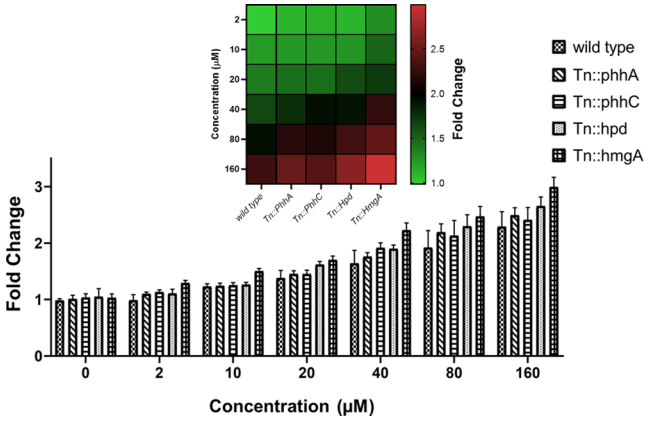

Figure 2. Fluorescence response of the strain carrying the phmgA::GFP plasmid was tested in the wild-type MPAO1 strain and transposon mutants in minimal media containing a mixture of amino acids at 4 h. The inset represents a heat map displaying the dose-dependent response of phmgA::GFP in wild-type and transposon mutant strains.

fluorescence expression of the *phmgA::GFP*-based biosensor for homogentisic acid detection. Of the four transposon mutants belonging to catabolism of the Phe/Tyr pathway, the Tn::hmgA mutant showed the best fluorescence response in terms of fold induction (Figure 2). A comparison between the fluorescence response of the wild-type MPAO1 strain and Tn::hmgA mutant shows an increase in the fluorescence intensity in response to homogentisic acid over the range of concentration 40−160 μM (Figure S4). Thus, due to its better sensitivity, Tn::hmgA was used as the biosensor chassis to assess the analytical features for the detection of homogentisic acid in simulated alkaptonuria samples. The transposon mutant of *hmgA* is expected to minimize the intracellular metabolism of homogentisic acid, leading to an increase in the fluorescence intensity.

Specificity and Interference Studies. To test the specificity of Tn::hmgA carrying biosensor plasmid *phmgA::GFP*, we examined the GFP response in the presence of 20 standard amino acids including Phe and Tyr (structural homologues of homogentisic acid) and homogentisic acid. Figure 3 represents the fluorescence response of 20 amino acids in PAO1, MPAO1, and Tn::hmgA carrying the *phmgA::GFP*-based biosensor construct. The Tn::hmgA mutant of the MPAO1 strain did not promote nonspecific fluorescence response from nontarget amino acids including structural homologues of homogentisic acid, thus confirming the specificity of the biosensor. Besides, Tn::hmgA also displayed a better fluorescence response to homogentisic acid when compared with PAO1 and MPAO1 strains (Figure 3).

Dose-Dependent Response of the Homogentisic Acid Biosensor. We investigated the dose-dependent relationship between the concentration of homogentisic acid and fold change in the fluorescence intensity of our sensor construct. Figure 4a represents the concentration-dependent response of the *phmgA::GFP* construct in Tn::hmgA transposon mutant MPAO1 chassis. The biosensor has a dynamic range extending from 10 to 320 μM (Figure 4a). We then sought to determine the linear correlation of our biosensor with varying concentrations of homogentisic acid. A calibration curve was generated over different concentrations of homogentisic acid. The fluorescence response presented a high degree of linearity (R^2 = 0.99) with the concentration of homogentisic acid ranging from 2 to 100 μM (Figure 4b). The limit of detection of the cell-based sensor was calculated to be 3.9 μM, which is comparable in sensitivity to other analytical techniques currently in use (Table S1).

Fluorescence Imaging and Flow Cytometry Analysis. Fluorescence imaging and flow cytometry analysis were performed to validate the biosensor performance as obtained through the fluorescence spectrophotometer at 4 h. Figure 4c represents fluorescence and DIC imaging of cells harboring the *phmgA::GFP* biosensing construct treated with 160 μM homogentisic acid. Our results show that the hmgA promoter element is tightly regulated by the hmgR protein since negligible background noise (in terms of GFP expression) was observed in uninduced cells as compared to the homogentisic acid-induced cells (Figure 4c). Flow cytometry results (Figure 4d) provide a quantitative estimation of homogentisic acid in terms of an increase in the fluorescence intensity of the bioreporter (*phmgA::GFP*). The inset represents a maximum peak shift in terms of fluorescence intensity at 160 μM (Figure 4d; inset). Results obtained through flow cytometry validate the spectrophotometric observations obtained from the biosensor strain during homogentisic acid detection.

Validation of the Homogentisic Acid-Based Biosensor. The precision and accuracy of the developed biosensor for the diagnosis of alkaptonuria were determined in the spiked human urine specimen. Besides, the results obtained by the cell-based assay were also compared to the conventional HPLC measurements. Table S2 lists the analytical results showing precision CVs (2.13−16.9) and accuracy (91.4−128.8), which are well within an acceptable range.[19,33,34] The precision and accuracy values demonstrate the reliability and reproducibility of the cell-based biosensor.[19,34,35] These results suggest that the cell-based fluorescence detection method can be a promising tool for the monitoring of homogentisic acid in alkaptonuria patients.

CONCLUSIONS

Elevation in the levels of homogentisic acid leads to black urine, which is considered as a hallmark of alkaptonuria. In this work, we report a novel whole-cell biosensor for direct determination of homogentisic acid, leading to the detection of alkaptonuria disease. The proposed biosensor displays excellent reproducibility, selectivity, and dynamic range (10−320 μM) for homogentisic acid quantification. Besides, the cell-based method overcomes the time-consuming, laborious, and technical steps like pre- and post-treatment required in conventional methods. The proposed biosensor can serve as a

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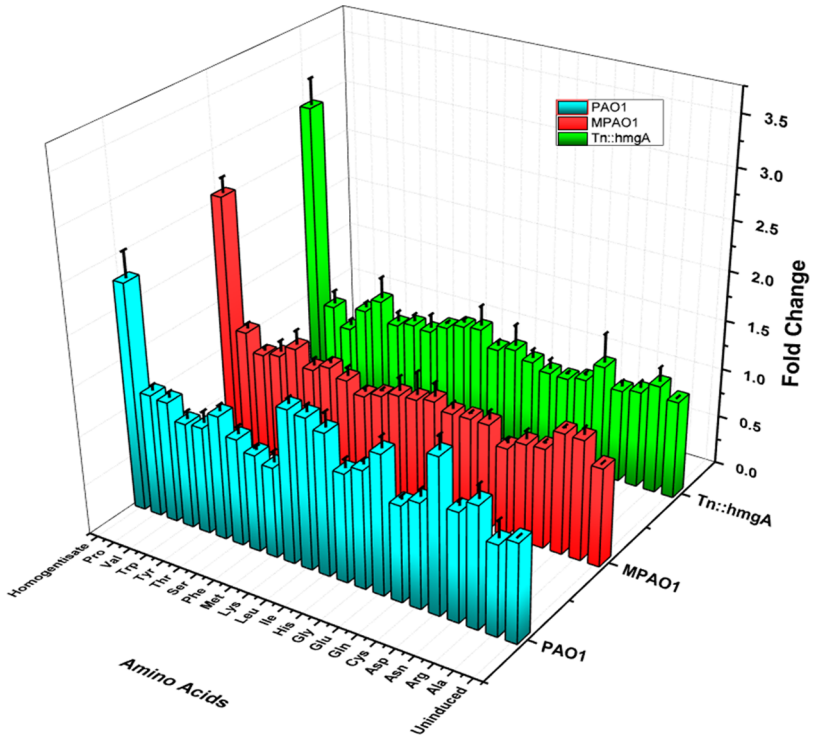

Figure 3. Selectivity and sensitivity of the proposed biosensor were recorded. Fluorescence activity of the strain carrying phmgA::GFP was examined in PAO1, MPAO1, and Tn::hmgA strains in response to 20 amino acids and homogentisic acid at 160 μM. The error bar represents standard deviation of the mean of three independent experiments. Results were analyzed using the one-way ANOVA test (****$P \leq 0.0001$).

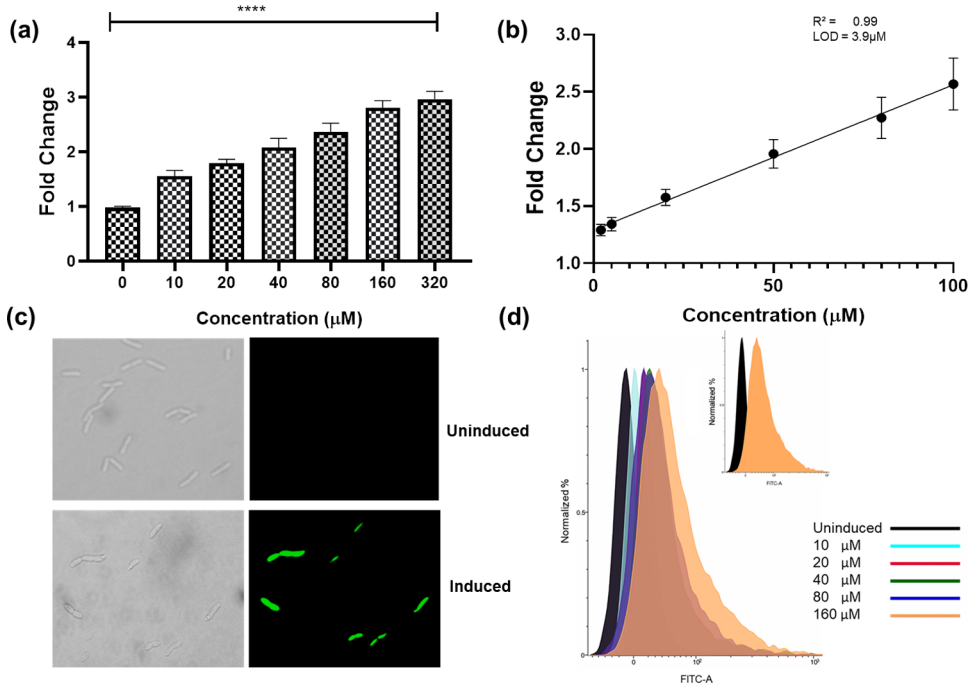

Figure 4. (a) Dose-dependent response of the strain carrying phmgA::GFP at 4 h in the hmgA transposon mutant (Tn::hmgA) strain in response to homogentisic acid. (b) Linear response of homogentisic acid was determined at a concentration range from 2 to 100 μM. The limit of detection (LOD) was calculated to be 3.9 μM. Error bars represent the standard deviation of the mean of three independent experiments. Results were analyzed with one-way ANOVA (****$P \leq 0.0001$). (c) Fluorescence and DIC microscopy of the homogentisic acid-based biosensor without homogentisic acid (uninduced) and with homogentisic acid (induced; 160 μM). (d) Validation of the phmgA::GFP biosensor by flow cytometry analysis. Dose-dependent response of the biosensor was analyzed over a range of 10−160 μM. The inset represents the peak shift in the fluorescence intensity at 160 μM concentration of homogentisic acid with respect to the uninduced case.

robust alternative to the conventional methods currently in use for the diagnosis of alkaptonuria disease. The biosensor demonstrates an improved limit of detection (3.9 μM) over other analytical techniques currently in use for homogentisic

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acid detection. Further studies could focus on genetic engineering approaches such as directed evolution of the promoter region to enhance the sensitivity for homogentisic acid and a faster response time. In addition to alkaptonuria, this biosensor may be useful to detect other pathological conditions such as colorectal cancer, ulcerative colitis, and Crohn's disease, where the homogentisic acid levels have been associated with the disease outcome.[36,37]

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.analchem.0c04914.

Design principle of the homogentisic acid biosensor, sensing module construction, optimization of fluorescence response, comparison of LOD with those of other analytical techniques, linear response and detection precision of homogentisic acid quantified through the whole-cell biosensor and HPLC-based methods, and bacterial strains used (PDF)

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Notes

The authors declare no competing financial interest.

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