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Development of a dimer-based screening system that targets PhoR, a sensor kinase of the two-component regulatory system, in *Mycobacterium tuberculosis*

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ABSTRACT The PhoR-PhoP two-component regulatory system, which is responsible for regulating the virulence of *Mycobacterium tuberculosis*, presents a promising target for the development of novel tuberculosis drugs. Disrupting the interaction of PhoR-PhoP proteins has the potential to decrease the virulence of the bacterium, rendering it more vulnerable to immune system clearance. A dimer-based screening system was developed to screen for inhibitors of PhoR dimerization. The coding sequence for the cytoplasmic domain of PhoR (cytoPhoR) was combined with the DNA-binding domain of the AraC repressor coding sequence. These sequences were positioned upstream of the emerald green fluorescent protein (EmGFP), which serves as a reporter gene. and controlled by the *araC* promoter. The in silico investigation examined the modeling of the fusion AraC_cytoPhoR and its binding to the promoter. The plasmid construct generated, namely pAraC_PhoRMTB, was synthesized and confirmed using DNA sequencing. The confirmed plasmid was then transformed into *Escherichia coli* BL21(DE3). Both SDS PAGE and fluorescence analysis indicated that the transformed culture expressed the AraC-cytoPhoR fusion protein and displayed lower relative fluorescence in comparison to the transformed culture consisting solely of the AraC DNA-binding domain coding sequence. This reduction in fluorescence suggests that the dimer-based screening system effectively monitors the inhibition of dimerization of cytoPhoR. These analysis findings indicate that the system is now ready for use in the screening of PhoR dimerization inhibitors.

KEYWORDS Dimerization inhibitor; *Mycobacterium tuberculosis*; PhoR; Screening; Two-component regulatory system

1. Introduction

Tuberculosis (TB) is a dangerous disease caused by *Mycobacterium tuberculosis* (Dhande et al. 2023). According to the Global TB Report released by World Health Organization (2022), Indonesia had the second highest number of TB cases in the world, with more than 700,000 incidents (Indonesia Ministry of Health 2023). In 2021, it was estimated that more than 1.6 million people around the world died due to TB, both with and without HIV co-infection (World Health Organization 2022).

Nowadays, most of TB infections are initiated from respiratory exposure, which takes place through bacteria-contaminated aerosol inhalation (Moule and Cirillo 2020). The initial site of infection may manifest pneumonia-like patches that can appear in any part of the lung, and which may progress to involve nearby lymph nodes, creating a collection of infections known as Ghon's complex. From there, bacilli will spread to the whole lung via surrounding lymph nodes. Starting with the lymph nodes, the infection can spread further into the bloodstream, resulting in a process called hematogenous dissemination. Hematoge-

nous reinfection of the lungs will result in secondary granulomas in the lungs' apical region or extrapulmonary site (Moule and Cirillo 2020).

M. tuberculosis possesses a heparin-binding hemagglutinin HbhA as an adhesin to attach to alveolar epithelial cells. The bacteria will then penetrate the epithelial cell, causing apoptosis or necrosis which will open the barrier of the alveolus (Moule and Cirillo 2020). *M. tuberculosis* can also live and multiply inside macrophages. It is theorized that *M. tuberculosis* disseminates throughout the body using macrophages which can cross into and out of the circulatory system (Moule and Cirillo 2020).

The typical treatment for TB involves administering antibiotics, but this approach faces the significant issue of resistance. Antibiotic resistance can occur for various reasons, such as non-compliant treatment or incorrect drug administration, while stronger antibiotics can cause adverse effects for the patient. An alternative treatment approach, distinct from antibiotics that target specific cellular components to induce cell death, is necessary to address resistance issues effectively.

The two-component regulatory system (TCS) is a set of proteins in microorganisms which functions by recognizing environmental changes and relaying that information into the cell so that the microorganism can generate an appropriate response to that change (Hirakawa et al. 2020). TCS consists of two proteins, histidine kinase (HK) which functions the sensor and signal deliverer into the cell, and a response regulator (RR) which functions by accepting signals from HK and regulating cell response to the environmental change (Hirakawa et al. 2020).

In *M. tuberculosis*, a very important TCS is PhoR (as HK protein) - PhoP (as RR protein), which regulates various genes including dormancy, hypoxia, and host immune modulation. It is estimated that the PhoR-PhoP system regulates more than 150 genes with various functions (Feng et al. 2018), such as it regulates genes in Region of Difference 1 (RD-1) which encodes ESAT-6 protein (Broset et al. 2015). ESAT-6/CFP-10 complex protein is secreted abundantly by *M. tuberculosis*, which functions to modulate the host's immune system (Sreejit et al. 2014). PhoR-PhoP also regulates lipid synthesis and export as cell wall components (Ryndak et al. 2008). Moreover, PhoR-PhoP TCS plays an important role in the low pH survival of *M. tuberculosis* in acidic macrophage vesicles or infection granulomas (Feng et al. 2018). Failure of the PhoR-PhoP system has been demonstrated to cause attenuation in *M. tuberculosis*, such as in avirulent strain H37Ra (Ryndak et al. 2008).

Impairing the PhoR-PhoP system also improves the elimination of the human immune system. *M. tuberculosis* lacking an intact PhoR-PhoP system exhibits a deficiency in sulfolipids (SL), diacyltrehalose (DAT), and polyacyltrehalose (PAT) lipids in the cell wall, compromising their role as inhibitors of the immune response (Queiroz and Riley 2017). These lipids interfere with the host immune system during infection. Moreover, a defective ESAT-6 secretion system results in the loss of the ability of *M. tuberculosis* to avoid phagosome and could possibly increase its immunogenicity due to the numerous human T-cell epitopes found in ESAT-6. A PhoR-PhoP mutant of *M. tuberculosis* also shows increased TAT substrate secretion, including antigens of the Ag85 complex, which are believed to result in an increased immune response (Broset et al. 2015).

It is also known that without functioning PhoR-PhoP TCS, c-di-AMP synthesis in *M. tuberculosis* would be unregulated, causing an increased innate immune response against the presence of the bacteria (Pérez et al. 2022). The isocitrate lyase (ICL) persistence factor plays a critical role in the acquisition by the *M. tuberculosis* metabolism of energy in the phagocytes of macrophage (Lee et al. 2015). It shows that TCS PhoR-PhoP is very important in *M. tuberculosis* pathogenesis and survival in its host. Therefore, the compounds that can inhibit the PhoR-PhoP system could potentially weaken *M. tuberculosis*, reducing its pathogenicity, and meaning that the bacteria can be cleared by the immune system more easily.

PhoR protein consists of an extracellular sensor domain, a transmembrane domain, and an intracellular (or cytoplasmic) domain. The cytoplasmic domain of PhoR has two functions, dimerization and ATPase activity which will phosphorylate PhoP protein (Ryndak et al. 2008). Dimerization of the PhoR cytoplasmic domain (cytoPhoR) is a very important step for PhoR as HK to transfer the phosphate group to PhoP as RR. PhoP will later bind to the phoP promoter to regulate various genes (Ryndak et al. 2008). This dimerization process can be used as a potential target for antitubercular agents. The screening for compounds that can inhibit the dimerization process of cytoPhoR can be conducted using the Dimer-Based Screening System (DBSS) adapted from Okada et al. (2007), utilizes the ability of HK to dimerize *in vitro* (Okada et al. 2007). The system is presented in Supplementary Figure 1.

The DBSS system has several advantages. First, it uses *Escherichia coli* which has a faster growing rate than *M. tuberculosis* so that the screening result will be faster. Second, using *Escherichia coli* is also safer compared to pathogenic *M. tuberculosis* meaning the system requires only a basic biosafety level laboratory. Therefore, the development of DBSS is important for rapid screening of new antituberculosis drug candidates.

The cytoPhoR coding sequence is fused with a DNA-binding domain (DBD) sequence from a repressor protein which must dimerize to bind with a promoter. A fluorescence protein gene is used as a reporter gene. In this research, the cytoPhoR coding sequence is fused with the DBD coding sequence of a repressor protein, upstream of an emerald green fluorescence protein (EmGFP) in pRSET vector. There are three plasmids with three different repressor-promoter pairs: repressor IclR – promoter *iclR* (Sundari et al. 2009; Kurnia 2012); repressor AraC – promoter *araC* (Rahmita 2015); and repressor Zif23 – promoter *zif23* (Fauziah 2015).

In this study, the three plasmid constructs were com-

pared, and the screening system optimized. The best plasmid construct was further optimized and tested for several organic compounds. The compound able to inhibit EmGFP repression, indicated by increased culture fluorescence intensity, was considered as a cytoPhoR dimerization inhibitor.

2. Materials and Methods

2.1. Selection of the best repressor-promoter pair

Glycerol stock transformant harboring the three plasmids with three different repressor-promoter pairs identified in previous research, repressor IclR – promoter *iclR* (Sundari et al. 2009; Kurnia 2012); repressor AraC – promoter *araC* (Rahmita 2015); and repressor Zif23 – promoter *zif23* (Fauziah 2015) were subcultured on Luria-Bertani (LB) agar plates with an ampicillin concentration of 100 µg/mL. The plates were incubated at 37 °C for 16 hours. A single colony was picked and inoculated into 10 mL LB broth with ampicillin 100 µg/mL, incubated at 37 °C and 200 rpm agitation overnight. A total of 5 mL of the culture was then inoculated into a new 50 mL LB broth with ampicillin 100 µg/mL, incubated at 37 °C and 200 rpm agitation, until the optical density at 600 nm wavelength (OD_{600}) reached 0.3. The culture was divided into two flasks, to which IPTG 1mM was added to one, but not the other. The culture was incubated at 37 °C and 200 rpm agitation for three hours. The OD_{600} of the culture was measured using a UV-Vis Shimadzu spectrophotometer (Japan), while its fluorescence was measured using a Shimadzu RF5301PC spectrofluorometer (Japan).

2.2. Plasmid construction optimization

Optimization of the plasmid construction is essential, given that the gene to be expressed originates from *M. tuberculosis*, while the expression will occur in *E. coli*. Various adjustments are necessary to facilitate the effective expression of the plasmid. The best plasmid construct, pRSET-araCsitoPhoR with repressor AraC – promoter *araC*, was optimized by codon optimization, and mRNA secondary structure optimization. The codon for the cytoPhoR coding sequence was also optimized to suit the host, *Escherichia coli* BL21(DE3). Codon optimization was performed using the Codon Optimization Tool from Integrated DNA Technologies (sg.idtdna.com/CodonOpt). Codon Adaptation Index calculation was made using CAIcal SERVER (http://ppuigbo.me/programs/CAIcal/), with codon reference from the Codon Usage Database (www.kazusa.or.jp/codon/cgi-bin/showcodon.cgi?species=413997). mRNA secondary structure optimization was performed using the RNAfold WebServer (http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi). Finally, optimized plasmid was synthesized by GenScript, USA. The optimized plasmid was named pAraC_PhoRMTB (Giri-Rachman et al. 2016), as shown in Figure 1.

2.3. pRSET-AraCDBD construction

pRSET-AraCDBD was constructed by removing the cytoPhoR part of the pAraC_PhoRMTB coding sequence. pAraC_PhoRMTB was cleaved using *Kpn*I and *Bam*HI restriction enzymes from ThermoScientific to remove the cytoPhoR coding sequence. The result was visualized using electrophoresis. The gel with a DNA band size of 4,229 base pairs was cut and purified using GenepHlow from Geneaid. The purified linear plasmid was then recircularized using T4 DNA ligase enzyme, with the result once again visualized using electrophoresis. The plasmid map of pRSET-AraCDBD is shown in Figure 1.

2.4. Plasmid transformation

A total of 10 µL of plasmid was added into 100 µL of competent *Escherichia coli* BL21(DE3) in a microtube and incubated in ice for 30 minutes. The microtube was then incubated at 42 °C for 1 minute. Subsequently, the microtube was incubated in ice for 5 minutes. A volume of 1 mL of LB broth was then added into the microtube, and the culture incubated at 37 °C and 200 rpm agitation for

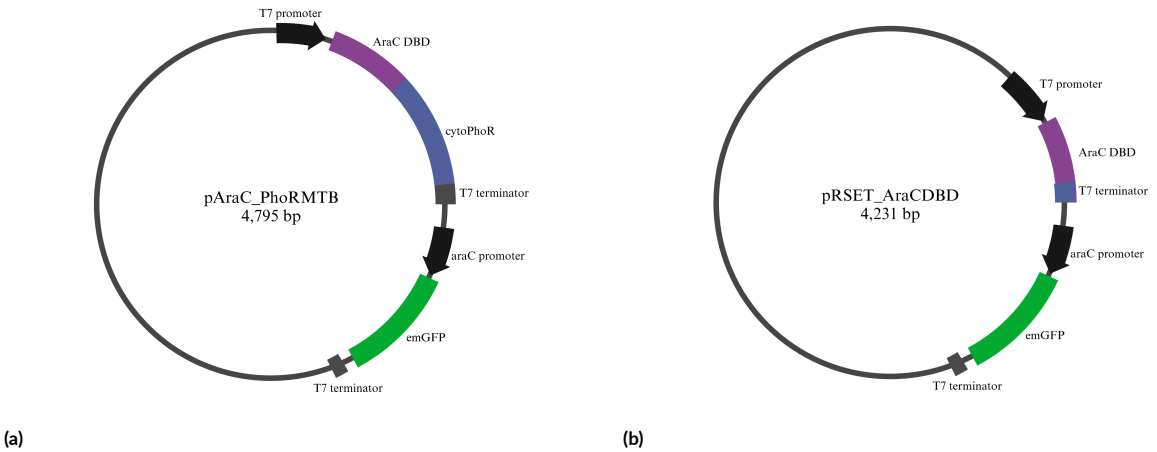

FIGURE 1 Constructed pAraC_PhoRMTB plasmid map (left) which was used in the screening process and pRSET-AraCDBD (right) which was used as a positive inhibition control in DBSS. Figure created with BioRender.com.

1 hour. Transformed culture was spread on the LB plate with ampicillin 100 µg/mL.

2.5. Protein expression analysis using SDS-PAGE

Overnight transformant culture with an OD_{600} of 0.4 was harvested using 14,000–16,000×g centrifugation for one minute. 100 µL SDS-PAGE loading buffer was added into the cell pellet. The mixture was then boiled for ten minutes. The stacking gel concentration was 5% and the separating gel concentration was 12%. SDS PAGE was run at 80V for 120 minutes. The gel was soaked in a staining solution overnight and then further soaked in destaining solution for 1–2 hours.

2.6. Dimer-based Screening System protocol

First, 1 mg of candidate compounds for novel TB drugs were dissolved in 100 µL of DMSO. A single colony of *Escherichia coli* BL21(DE3) harboring pAraC_PhoRMTB was then inoculated in 10 mL LB broth with ampicillin 100 µg/mL and then incubated at 37 °C and 200 rpm overnight. Subsequently, 0.5 µL of the culture was transferred to 97 µL LB broth with an ampicillin concentration of 100 µg/mL and 2.5 µL tested compound inside a 96-well plate. Eight different compounds were tested. pAraC_PhoRMTB transformant culture added with DMSO only was used as a dimerization inhibition negative control and pRSET-AraCDBD transformant culture added with DMSO only used as a dimerization inhibition positive control. Measurement of fluorescence intensity (excitation wavelength of 475 nm, emission wavelength of 500–550 nm) and culture biomass at OD_{600} was made using Glomax Discover microplate reader. Normalization of the sample fluorescence intensity was calculated relative to the culture biomass.

2.7. 3D Structure Modeling and Molecular Docking

3D structure modeling of AraC-cytoPhoR fusion protein was performed using AlphaFold2 on the ColabFold AF2-multimer server based on Google Colab called "AlphaFold2.ipynb". Docking of AraC-cytoPhoR fusion protein with DNA-promoter was performed by HDOCK, while molecular interaction was evaluated by a knowledge-based iterative scoring function. The fusion protein dimer of AraC-cytoPhoR was modeled using the symmetric docking RosettaDock modeling suite (http://www.rosettacommons.org), which is a fast conformational searching protein that is suitable for modeling protein dimer. The optimal model was subsequently chosen and rendered using Chimera X-1.61 ((Pettersen et al. 2021). Validation of the model structure was conducted by utilizing PROCHECK, which assessed the torsional angles via a Ramachandran plot (Laskowski et al. 1993).

3. Results and Discussion

M. tuberculosis is known to have a slow growth rate, which can reach days on Lowenstein-Jensen medium. Moreover, it is airborne and highly contagious. These problems have hindered the advancement and discovery of much-needed new therapy in the midst of growing drug-resistant TB cases. Using the *Escherichia coli* expression system, which is quicker to culture and much safer to handle, DBSS provides a solution to these obstacles.

Previously, three DBSSs have been successfully constructed, which are repressor IclR – promoter *iclR* (Sundari et al. 2009; Kurnia 2012); repressor AraC – promoter *araC* (Rahmita 2015); and repressor Zif23 – promoter *zif23* (Fauziah 2015). All three repression systems are naturally found or synthetically engineered for *E. coli* cells. The IclR repression system plays a role in the glyoxylate cycle to regulate the *aceBAK* promoter (Lai et al. 2021), while the AraC repressor protein binds to operator O2 and I1 and regulates the L-arabinose catabolism operon (Bhat et al. 2023). The Zif23 repression system is a syn-

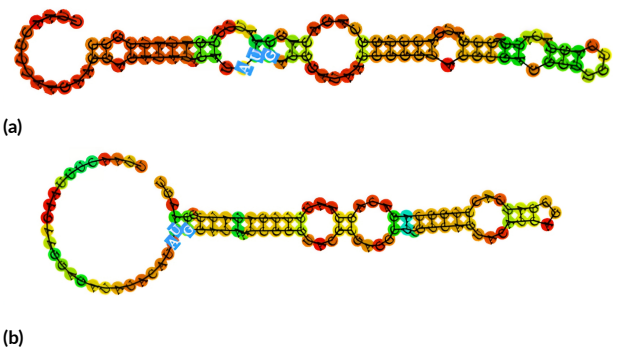

FIGURE 2 Predicted mRNA secondary structure using RNAfold WebServer, (a) before and (b) after optimization. Before optimization, the predicted gene fusion transcript was shown to be tightly packed, with numerous red or orange nucleotides in the pairing regions, indicating that the probability of self-pairing was high in those regions. After optimization, the predicted gene fusion transcript was shown to be more open, with fewer red nucleotides in the pairing regions, indicating that the probability of self-pairing was lower (ViennaRNA Web Services n.d.).

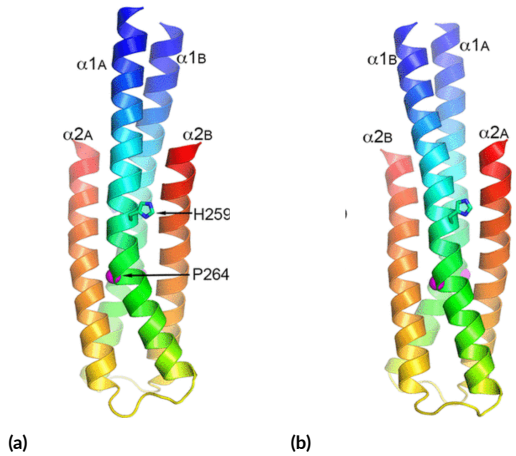

FIGURE 3 Images of cytoPhoR dimerization from Xing et al. (2017). Two subunits, A and B, are shown with (a) front and (b) back views. The phosphorylation H259 residue is shown as sticks, whereas the P264 residue is shown as a magenta ball. The kink in subunit A is more prominent than in subunit B, which creates an asymmetric dimer for cytoPhoR.

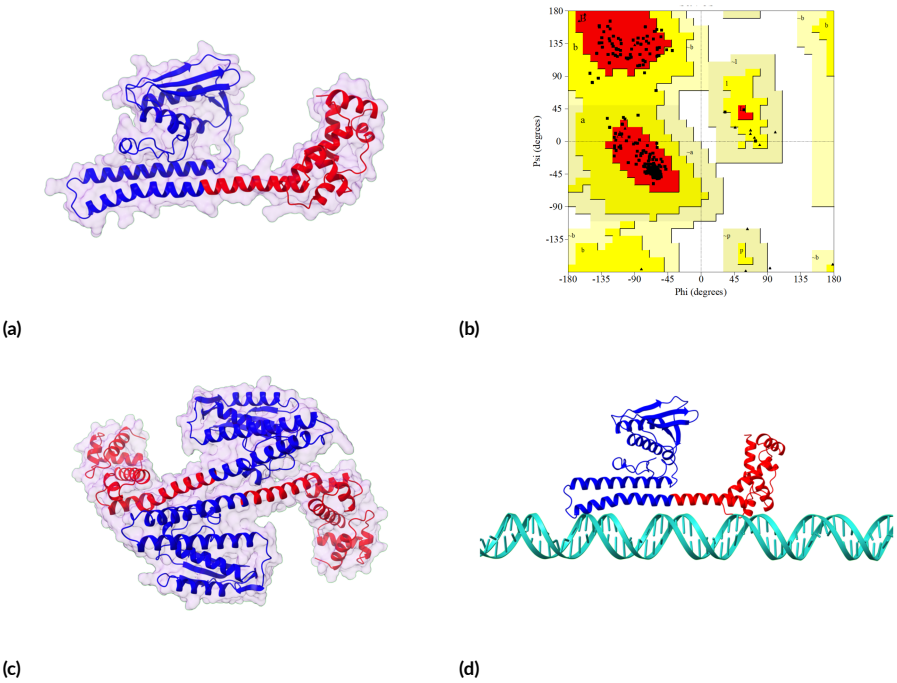

FIGURE 4 (a) Three-dimensional structure model of AraC-cytoPhoR fusion protein and (b) Ramachandran plot of 3D model structure. The DNA-binding domain of AraC is highlighted in red, and the cytoPhoR domain is represented in blue. (c) Dimeric model of AraC-cytoPhoR. The DNA-binding domain of AraC is highlighted in red and the cytoPhoR (dimerization) domain in blue. (d) Docking of AraC-cytoPhoR fusion protein with *araC* promoter, showing that binding of the fusion protein with the promoter sequence is possible.

thetic system based on the eukaryotic Zif268 repression system from *Mus musculus* and *Saccharomyces cerevisiae* (Fauziah and Giri-Rachman 2016).

The fluorescence analysis result of three transformant cultures are shown in Figure 5a. When the repressor is induced to be expressed, repressor protein will be produced. As previously stated, when no homodimerization inhibitor is present, the repressor protein will form a homodimer and bind to the promoter of the EmGFP reporter gene, thus lowering fluorescence intensity. On the contrary, a non-induced culture will produce no repressor protein and thus fluorescence intensity will increase. The results showed that repressor AraC – promoter *araC* produced the biggest difference between IPTG induced and non-induced culture compared to repressor IclR – promoter *iclR* and repressor Zif23 – promoter *zif23*. This result is in accordance with Balzer et al. (2013), who showed that *araC* is a strong promoter and that AraC repressor has a strong repression capability inside the cell, even in low numbers.

TABLE 1 Interaction residues of AraC-cytoPhoR and *araC* DNA Promoter.

AraC-cytoPhoR Protein	***araC* DNA Promoter**
AraC-region:	**Strand A (5′):**
69, 71–72, 84, 100, 103–105, 107, 114–115, 118–119	90–91, 99–101, 108–109
cytoPhoR region:	**Strand B (3′):**
121–123,125–126, 136, 140, 143–144, 147	174–177, 182–185, 192–194

The higher standard deviation observed in the AraC-sitoPhoR transformant culture compared to the others is plausibly due to cell density. The strong repression capability of the AraC repressor could also contribute to greater variability in these results, as even small differences in repressor expression levels or induction efficiency can lead to significant changes in fluorescence. This phenomenon was not observed in IclR-cytoPhoR and Zif23-cytoPhoR.

The repressor IclR - promoter *iclR* pair was previously used by Okada et al. (2007), on whose work this research is based. In our study, it was found that the AraC repression system was better than the IclR system in terms of regulating the EmGFP reporter gene. A greater fluorescence difference between induced and non-induced culture will make it clearer to differentiate between inhibition and no inhibition conditions, which is preferable in this research. Therefore, the plasmid with the repressor AraC – promoter *araC* pair was selected to be optimized and used in the Dimer-Based Screening System.

Several optimizations of the plasmid were employed for this research: first, optimization of the mRNA secondary structure of the fusion protein. This optimization was intended to prevent the mRNA produced by the plasmid from creating a secondary structure that will hinder ribosome attachment which is crucial for gene expression. The mRNA structure before and after optimization is shown in Figure 2.

As can be seen in the figure above, the mRNA sequence upstream of the start codon is more open after optimization, which increases the accessibility of the transcript to ribosomes (Figure 2b). Second, codon optimization

was performed on the cytoPhoR coding sequence because the sequence was taken from *M. tuberculosis* H37Rv and would be expressed in *Escherichia coli* strain BL21. The codon optimization process increased the Codon Adaptation Index (CAI) from 0.674 to 0.885, meaning that the PhoR gene of *M. tuberculosis* would have a greater probability of being easily expressed by the *Escherichia coli* expression system. Third, addition of T7 terminator downstream of the fusion protein coding sequence was made to stop the transcription.

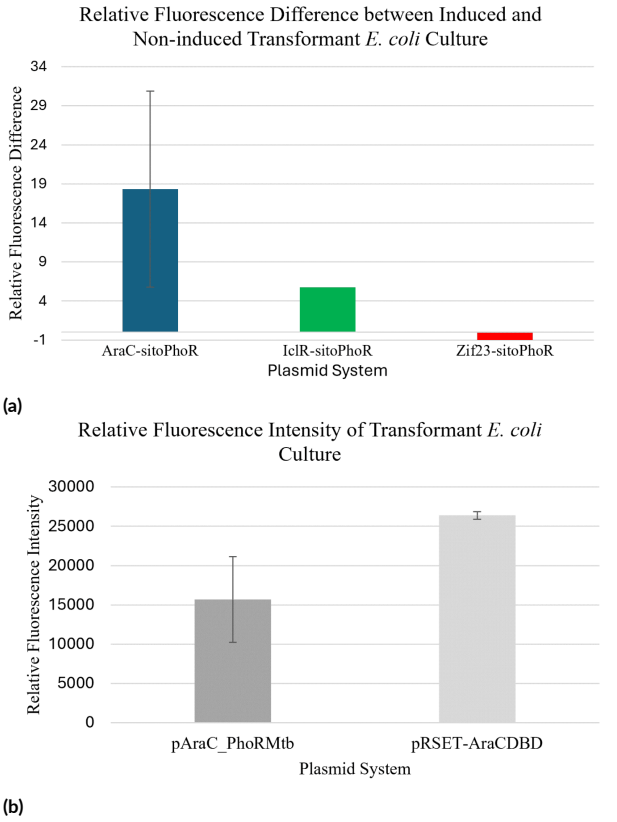

FIGURE 5 (a) Relative fluorescence difference between repressor-induced and non-repressor-induced transformant *E. coli* culture. AraC and IclR repressors are present in *E. coli* cells, while Zif23 repressor is a synthetic repressor for the *E. coli* expression system. (b) Fluorescence comparison between pAraC_PhoRMTB transformant *Escherichia coli* BL21(DE3) culture and pRSET-AraCDBD transformant *Escherichia coli* BL21(DE3) culture. The pRSET-AraCDBD system, which contains no dimerization domain gene of cytophoR, was used as the positive control of dimerization inhibition. In a condition of positive inhibition, the system produced a higher intensity of fluorescence compared to the negative inhibition control (pAraC_PhoRMtb system).

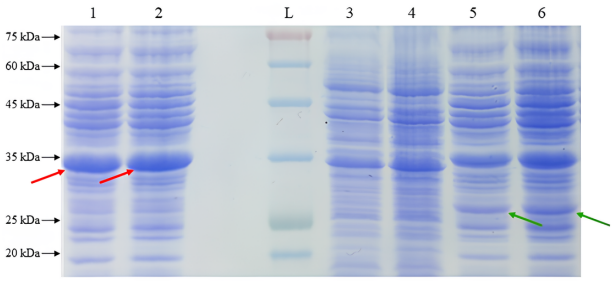

FIGURE 6 SDS-PAGE results of (from left to right) 1: pAraC_PhoRMtb transformant culture without IPTG; 2: pAraC_PhoRMtb transformant culture with IPTG; L: Smobio PM2600 protein ladder; 3: empty *Escherichia coli* BL21(DE3) without IPTG; 4: empty *Escherichia coli* BL21(DE3) with IPTG; 5: pRSET-EmGFP transformant culture without IPTG; and 6: pRSET-EmGFP transformant culture with IPTG. Green arrow: presumed EmGFP protein (26.9 kDa); red arrow: presumed AraC-cytoPhoR fusion protein (33.8 kDa).

The central region of the CytoPhoR domain consists mainly of strongly hydrophobic amino acids. This region plays an important role in the dimerization process, as it holds the protein bundle with hydrophobic interactions. The protein structure is flexible and can form an asymmetric homodimer due to a kink in one of the proteins caused by proline 264 (Xing et al. 2017). The structure of the cytoPhoR homodimer from Xing et al. (2017) is shown in Figure 3.

In silico modeling and analysis of the fusion protein, its dimerization, and the dimer interaction with araC promoter was performed to better understand the molecular interaction and validate the screening system. In this study, we utilized AlphaFold to generate a 3D structural model for the fusion protein AraC-cytoPhoR, subsequently evaluating the quality of the model through PROCHECK. The analysis of the Ramachandran Plot revealed that 94.2% of the torsional angles in the AraC-cytoPhoR model were located within favorable regions, as illustrated in Figure 4a and b.

As previously explained, non-polar (hydrophobic) amino acids are present in sufficient quantities to predominate in the AraC-cytoPhoR dimer interface. These hydrophobic interactions are essential in the dimerization process. In addition, the formation of dimers is also strengthened by the presence of electrostatic interactions from charged residues. The AraC-cytoPhoR dimer model is shown in Figure 4c.

The fusion protein was also docked with *araC* promoter to predict its ability to bind with the promoter sequence and inhibit the expression of EmGFP, as illustrated in Figure 4d. The resulting docking score was -190.84 with a confidence score of 0.6936, suggesting plausible binding potential between the two molecules. Based on the analysis of the interaction, it was observed that the helix formed by the conjunction of AraC and cytoPhoR interacts with the *araC* DNA promoter. Detailed enumeration of the residues involved in this interaction is provided in Table 1.

The optimized plasmid was synthesized by GenScript, USA, and the plasmid was transformed into *Escherichia coli* BL21(DE3). Plasmid confirmation was performed using the DNA sequencing method. Alignment of the sequenced plasmid and reference plasmid showed 100% identity, which indicated that the plasmid was error-free and could be used in the screening.

Since no currently available drug has been proven to inhibit the dimerization of cytoPhoR in *M. tuberculosis*, a different approach for positive control is needed for DBSS. Another plasmid used in this research was

pRSET-AraCDBD, which was created by removing the cytophoR gene from pAraC_PhoRMTB. Therefore, no dimerization domain was available, and repression of the reporter genes would not be possible. This is regarded as equivalent to the positive inhibition of cytoPhoR dimerization control. We conducted a fluorescence comparison between pAraC_PhoRMTB transformant culture and pRSET-AraCDBD transformant culture; the results are shown in Figure 5b. The relative fluorescence of pAraC_PhoRMTB transformant culture was lower than that of pRSET-AraCDBD. The *E. coli* culture harboring the pAraC-PhoRMTB plasmid continued to exhibit fluorescence, which was probably attributable to the inherent leakiness of the *araC* promoter, which is a native expression system in *E. coli* cells. The error bar of both charts does not overlap, which shows that the difference is statistically significant. This indicates that the repression by the fusion protein AraC-cytoPhoR was successful, and that the system was ready to be used in the screening process.

We also analyzed the protein expression of the transformant culture using SDS PAGE. The SDS-PAGE analysis shown in Figure 6 reveals a distinct band at around 33 kDa (highlighted by the red arrow) in the pAraC_PhoRMTB transformant culture, suggesting the presence of the AraC-cytoPhoR fusion protein. Notably, in the presence of this band, the EmGFP reporter protein was undetectable, as opposed to the pRSET-EmGFP transformant culture, in which a prominent EmGFP band was observed (indicated by the green arrow). This finding provides additional confirmation of the successful construction of the repression system. It should be noted that the T7 promoter regulating the araC-cytophoR gene exhibited leakiness and did not necessitate IPTG induction.

We then attempted to test the screening system using eight organic compounds from the Organic Chemistry Research Group Bandung Institute of Technology library. Organic compounds were chosen because of their potential antimicrobial activity. The results of the screening are shown in Figure 7.

Among the candidate compounds, Compound-2, a derivative of cinnamic acid isolated from black cumin, exhibited the highest relative fluorescence, at 200 µg/mL, while also demonstrating a linear relationship between fluorescence intensity and concentration. This suggested that Compound-2 may hinder cytoPhoR dimerization, preventing the repression of EmGFP in the plasmid, thereby accounting for the observed 36.31% increase in fluorescence. In contrast, Compound-1-6 and 7 (flavonoid derivatives), Compound-8 (sesquiterpenoid derivative), and Compound-1, 3, 4, and 5 (chalcone derivatives) did not significantly impact the dimerization ability of cytoPhoR or inhibit EmGFP.

Compound-2 possesses a relatively long carbon chain, rendering it the most non-polar among all tested compounds. As previously discussed, the prevalence of non-polar (hydrophobic) amino acids in the AraC-cytoPhoR dimer interface underscores the significance of these hydrophobic interactions in the dimerization process. Therefore, we propose that Compound-2 is more likely to strongly bind to this hydrophobic interface, potentially serving as an inhibitor of AraC-cytoPhoR.

The development of the Dimer-Based Screening System has demonstrated its versatility and effectiveness in identifying promising novel drug candidates targeting specific dimeric proteins within two-component regulatory systems. Following the screening process using DBSS, the identified tuberculosis drug candidates will undergo further evaluation against live M. tuberculosis cultures. This comprehensive approach holds significant promise for advancing the development of much-needed treatments for drug-resistant tuberculosis.

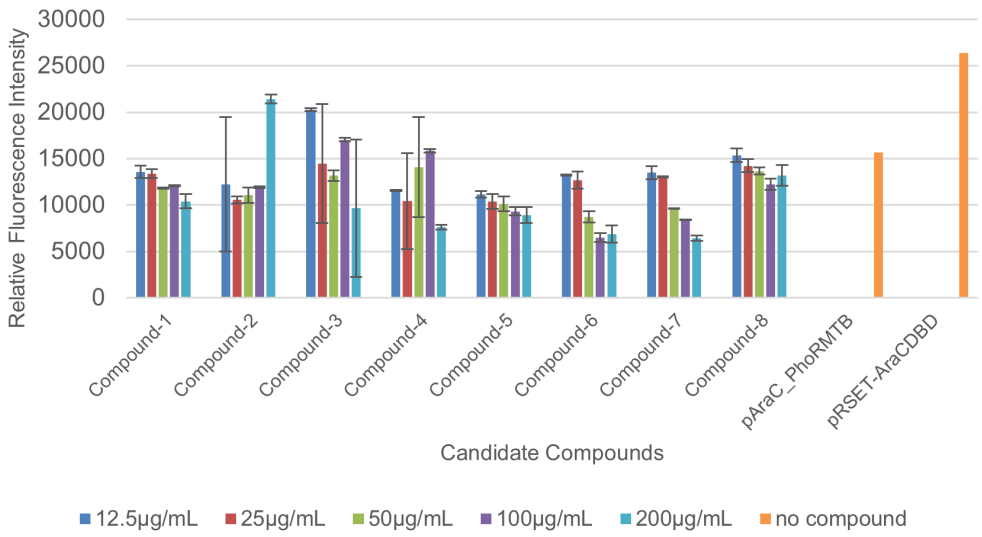

FIGURE 7 Relative fluorescence of the eight organic compounds using the Dimer-Based Screening System. Both negative inhibition (pAraC_PhoRMTB) and positive inhibition (pRSET_AraCDBD) controls worked well in the testing.

4. Conclusions

The Dimer-Based Screening System offers the capability to identify compounds with the potential to inhibit the dimerization of cytoPhoR, making them promising candidates for the development of novel antituberculosis drugs. Additionally, this versatile system can be employed for drug screening targeting two-component regulatory systems (TCS) or other diseases. Beyond drug discovery, TCS demonstrates significant potential as sensors for diverse use in synthetic biology. Hence, the applications of this system extend beyond drug discovery, showcasing its versatility in various scientific domains.

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Authors' contributions

EAGR and NS designed the study. RA and AA provided the in-silico study. YMS, EH and EB provided natural compounds for the experiments. NS carried out the laboratory work. NS, EAGR, and AF analyzed the data. NS and EAGR wrote the manuscript. All the authors read and approved the final version of the manuscript.

Competing interests

The authors declare that there are no conflicts of interest related to this publication

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