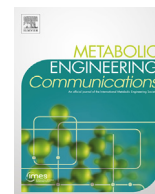


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CRISPR/Cas9-facilitated engineering with growth-coupled and sensor-guided *in vivo* screening of enzyme variants for a more efficient chorismate pathway in *E. coli*

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ABSTRACT

Protein engineering plays an increasingly important role in developing new and optimizing existing metabolic pathways for biosynthesis. Conventional screening approach of libraries of gene and enzyme variants is often done using a host strain under conditions not relevant to the cultivation or intracellular conditions of the later production strain. This does not necessarily result in the identification of the best enzyme variant for *in vivo* use in the production strain. In this work, we propose a method which integrates CRISPR/Cas9-facilitated engineering of the target gene(s) with growth-coupled and sensor-guided *in vivo* screening (CGSS) for protein engineering and pathway optimization. The efficiency of the method is demonstrated for engineering 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) synthase AroG, a key enzyme in the chorismate pathway for the synthesis of aromatic amino acids (AAAs), to obtain variants of AroG (AroG^{fbt}) with increased resistance to feedback inhibition of Phe. Starting from a tryptophan (Trp)-producing *E. coli* strain (harboring a reported Phe-resistant AroG variant AroG^{S180F}), the removal of all the endogenous DAHP synthases makes the growth of this strain dependent on the activity of an introduced AroG variant. The different catalytic efficiencies of AroG variants lead to different intracellular concentration of Trp which is sensed by a Trp biosensor (TnaC-eGFP). Using the growth rate and the signal strength of the biosensor as criteria, we successfully identified several novel Phe-resistant AroG variants (including the best one AroG^{D6G-D7A}) which exhibited higher specific enzyme activity than that of the reference variant AroG^{S180F} at the presence of 40 mM Phe. The replacement of AroG^{S180F} with the newly identified AroG^{D6G-D7A} in the Trp-producing strain significantly improved the Trp production by 38.5% (24.03 ± 1.02 g/L at 36 h) in a simple fed-batch fermentation.

1. Introduction

Microbes have been extensively engineered to produce value-added compounds from renewable feedstock (Becker et al., 2015; Lee and Kim, 2015; Liao et al., 2016). For this purpose, the engineering of biosynthetic pathways plays a crucial role. Several strategies based on the adjustment of gene expression and enzyme concentrations have been developed (Alper et al., 2005; Blazeck and Alper, 2013; Hwang et al., 2018; Zhou and Zeng, 2015). However, they are not able to overcome some inherent limitations associated with the enzymes themselves. For instance, regulation mechanisms of enzyme such as feed-back or feed-forward inhibitions (Chen et al., 2018; Zurawski et al., 1981)

restricting the catalytic efficiency. In this regard, the construction of an efficient pathway inevitably requires dedicated protein engineering (Chen et al., 2011b, 2013; Ger et al., 1994; Lin et al., 2012).

Normally, protein engineering consists of three distinct steps: (i) construction of a gene variant library; (ii) screening of the library and (iii) further characterization of candidate enzyme variants (Boville et al., 2018; Buller et al., 2018) (Fig. 1b). It has been widely and successfully used for improving the performance of many enzymes (Chen et al., 2011a; Rees et al., 2017; Sun et al., 2016). However, due to several limiting factors, enzyme variants obtained from conventional protein engineering approaches may be phenotypically hardly evaluable in the screening host, making further identification of desired enzyme variants

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rather challenging and time-consuming (Ren et al., 2018). For instance, insufficient phenotypic characterization, especially that based solely on cell growth rate, makes it hard to identify the best performer among the apparently improved enzyme variants (Ren et al., 2015). Furthermore, the expression of variants on plasmid is usually controlled with a strong promoter or with a high copy number. This makes it inefficient to filter out variants with relatively low activities, making further evaluation of the variants laborious. Also the subsequent *in vitro* and *in vivo* characterizations of the best performer identified from screening may not be relevant to a real bioproduction process using the host microorganism. For these reasons, it is of great interest to develop an approach that combines the integration of gene variants of a targeted enzyme directly into the chromosome of the host microorganism with *in vivo* screening and characterization of the enzyme variants under conditions more relevant to the cultivation and intracellular environmental conditions of a host strain to be used for the bioproduction process. To our best knowledge, no such an integrated approach has been reported so far.

Recently, the CRISPR/Cas9 technology for genome editing has made great advances and received large attention (Cho et al., 2018; Donohoue et al., 2017; Jakočiūnas et al., 2015; Zhang et al., 2018). Among others, it is applied for the engineering of microbial production strains (Jakočiūnas et al., 2015; Jiang et al., 2015; Schuster et al., 2018). Due to its simplicity and efficiency, CRISPR/Cas9 is an ideal genome-editing tool for quickly and effectively integrating gene variants of a target enzyme into the chromosome of a production strain (Guo et al., 2018). To cope with this technology, a reliable *in vivo* screening method suitable for fast screening is desired. In this regard, it is desirable to link the change of interest caused by an induced mutation on a target gene to a change in cell growth (Lu et al., 2012; Zhu et al., 2017) or to the expression strength of a reporter gene via a biosensor (Binder et al., 2013; Fang et al., 2016) or ideally to both of them combined. Using cell growth rate alone often makes it hard to identify the best performer among the improved enzyme variants (Ren et al., 2015), while using a biosensor alone requires a high throughput screening equipment, like FACS, which is not economically available in every laboratory. These limitations are likely to be overcome by using cell growth and biosensor together. In this study, a method of linking CRISPR/Cas9-facilitated engineering with growth-coupled and sensor-guided *in vivo* screening (CGSS) is proposed for protein engineering (Fig. 1).

Generally, using CGSS, it's able to effectively evaluate variants of the target gene with a relatively low and genetically stable expression in the chromosome of a strain that is to be further developed as the production host. *In vitro* constructed mutation library of the target gene can be

integrated into the chromosome using lambda-red recombination coupled with the CRISPR/Cas9 system for a higher efficiency. To make the mutation of the target gene position-independent, an expression cassette with a marker gene, usually an antibiotic-resistant gene, is used to replace the endogenous target gene and to offer sgRNA targeting sites. To maximize the effectiveness and reliability of this method, the growth of the selected host is linked to the efficiency of the gene variant in catalyzing the metabolic pathway leading to the formation of the targeted product, the concentration of which in turn controls the expression of a reporter through a biosensor. In short, favorable variants will be selected according to the cell growth and the expression of the reporter gene indicating the concentration of the targeted product.

To demonstrate this method, the optimization of a key enzyme of the chorismate pathway for the biosynthesis of L-tryptophan (Trp) was performed as an example. 3-Deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) synthase is a key enzyme for engineering microbes for efficient biosynthesis of aromatic amino acids (AAAs): Trp, phenylalanine (Phe), and tyrosine (Tyr) (Chen and Zeng, 2017; Kim et al., 2015; Wu et al., 2018). It is under strong feedback inhibition by the end products (Ogino et al., 1982; Sprenger, 2006). For instance, in *E. coli*, all the wild-types of DAHP synthase, encoded by the genes *aroG*, *aroF*, and *aroH*, are subject to feedback inhibition by Phe, Tyr, and Trp, respectively (McCandliss et al., 1978; Schoner and Herrmann, 1976). Therefore, the engineering of feedback-resistant DAHP synthase is indispensable to the construction of efficient pathways for producing AAAs and their derivatives (Sprenger, 2006). In this study, CGSS is proposed and demonstrated for semi-rationally engineering and screening feedback-resistant DAHP synthase to have a more efficient chorismate pathway for Trp production in *E. coli*. To implement the CGSS approach, first, the chorismate pathway in the host strain (S028Δ*aroH*Δ*aroF*Δ*aroG*^{S180F}) should be blocked by knocking-out the reference *aroG*^{S180F} gene and replaced with the antibiotic gene *CmR*. Then the gene *CmR* was replaced with variants of the whole gene *aroG* by using the CRISPR-Cas9 system. In such a way, it can be made sure that the growth rate of the host strain is directly coupled to the enzyme activity of the AroG variant. Moreover, if the genome encoded *aroG*^{S180F}-variant is targeted directly using the CRISPR-Cas9 system, there might have been more false positives (*AroG*^{S180F}-variant) since off-target recognition can happen in the CRISPR-Cas9 system. It is noted that the growth-coupled and sensor-guided library screening approach demonstrated in this work can be combined with *in vivo* targeted mutagenesis (Camps, 2018; Jakočiūnas et al., 2018; Ravikummar et al., 2018). This would result in an even more advanced approach with deep mutation and screening of enzymes.

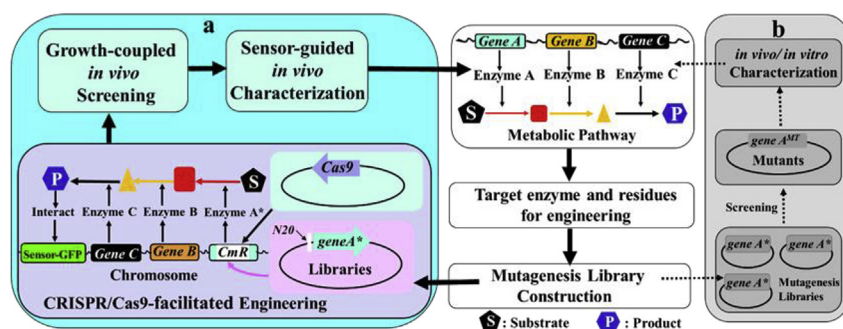


Fig. 1. (a) CRISPR/Cas9-facilitated engineering of gene variants integrated with growth-coupled and sensor-guided *in vivo* screening of candidate enzyme(s) as a novel approach (CGSS) for protein and pathway engineering. (b) Conventional screening and characterization approach of protein engineering. The middle part illustrates the enzyme(s) in the context of pathway engineering and the identification of key enzyme(s) and residues for the construction of mutagenesis library. It is shared by the two approaches. The major advantages of CGSS lie in the integration of the gene variants (genotype) directly into the chromosome of the production strain and *in vivo* evaluation (screening and characterization) of the variants through the phenotype such as cell growth rate and concentration of the relative metabolite using a biomolecular sensor. This can be done under cultivation conditions related to the real use of the engineered enzyme and the corresponding production strain. The conventional approach normally involves expression (e.g. using plasmid) of the mutagenesis library in a host differently from the production strain, *in vitro* and *in vivo* screening and characterization under conditions which have little to do with the real culture and intracellular environmental conditions of the later production strain.

2. Materials and methods

2.1. Strains and plasmids

The strains and plasmids used in this study are listed in Table 1. A Trp producing strain S028 (Chen and Zeng, 2017) was used as the starting strain. For unknown reason(s) the λ -red recombination system contained in the plasmid pCas (Jiang et al., 2015) had a quite low efficiency in the strain S028. Thus, the parent strain S028 λ which chromosomally containing the temperature-sensitive λ -red recombinering system was used. In the strain S028 λ , the DAHP synthase activity is conferred only by the Phe-resistant AroG^{S180F}. To use the CRISPR/Cas9 technique for genome-editing, the plasmid pCas (Jiang et al., 2015) was introduced into S028 λ , resulting in the strain S028 λ C (Table 1).

To construct plasmids for protein expression and purification, the encoding gene of aroG^{WT} was isolated from the strain *E. coli* DY330 (Yu et al., 2000) with primers *aroG-His-HindIII* and *XbaI-serA* (Table S1). It was then inserted into the vector pET22b(+) at the sites *HindIII* and *XbaI* to generate the plasmid pET-AroG^{WT}. The plasmids pET-AroG^{S180F}, pET-AroG^{D6G-D7A}, pET-AroG^{D6L-D7P}, and pET-AroG^{D6P-D7I} were generated by using corresponding mutagenic primers (Table S1) to amplify the whole plasmid pET-AroG^{WT}. More specific genetic work for strain development is described below.

2.2. Implementation of CRISPR/Cas9 facilitated engineering and screening

2.2.1. Construction of the host for in vivo screening and the donor DNA plasmid for library preparation

To realize the new approach proposed in this work, we first constructed an auxotrophic strain for aromatic amino acids. It contains a Trp biosensor but lacks DAHP synthase activity for screening AroG variants. First, the Trp-biosensor (*P_{tac}-tnaC-eGFP*) was integrated into the chromosome of a previously developed Trp-producing strain S028 λ C at the

locus of the *trpR* gene using the CRISPR/Cas9 system, resulting in the strain WS001 (Table 1). Then, we removed the sole DAHP synthase of the strain WS001 by replacing the *aroG*^{S180F} gene with the antibiotic resistance gene *CmR* which was used as a selection marker for gene editing and offers single guide RNA (sgRNA) targets for the CRISPR/Cas9 system for further genome-editing, generating the strain WS002 (Table 1, Fig. 2). To achieve a high efficiency for genome editing, the plasmid pCm-aroG^{WT} (Fig. S1) was constructed. This plasmid is able to express the sgRNA targeting to the *CmR* gene and the donor DNA containing the *aroG* gene and the homologous arms for recombination. It was used as a template for preparing the mutation library of *aroG* by PCR. More detailed information for the construction of pCm-aroG^{WT} is presented in the Supplementary Material.

2.2.2. Genome editing with CRISPR/Cas9 technique

As mentioned above, the λ -red recombination system contained in the plasmid pCas works poorly in our strains for unknown reason(s). Therefore, the chromosomal-integrated λ -red recombination system in the strain S028 λ C was used in this study. We then followed the protocol reported by Chen and Zeng (2017) to prepare electroporation-competent cells and to do the transformation. Specifically, an overnight culture (grown at 30 °C) of the strain S028 λ C was inoculated (2%, vol/vol) into 10 ml fresh SOB medium containing 30 μ g/ml kanamycin. After OD600 reaching to 0.4–0.5, the λ -red recombination system was induced by incubating the cells at 42 °C for 15 min under shaking. The cells were then put on ice immediately for 10 min. After that, the cells were harvested by centrifugation at 4 °C and washed three times with precooled 10% glycerol or distilled water. Competent cells were re-suspended in 400 μ l precooled 10% glycerol and divided into 200 μ l for each reaction. The corresponding sgRNA plasmid eluted in water was mixed with the competent cells for transformation. The electroporation was done in the 0.2 cm cuvette at 2.5 kV, and the cells were suspended in 1 ml SOB medium and recovered for 2 h at 30 °C before plating. Plates were incubated

Table 1

Main strains and plasmids used in this study.

Strains/Plasmids	Characteristics	Sources
Strains		
DY330	<i>E. coli</i> K-12 W3110 Δ lacU169 gal490 λ CI857 Δ (cro-bioA)	Yu et al. (2000)
S028	DY330 <i>rpsL</i> (StrR) Δ mtt Δ tnaA Δ tnaB Δ l Δ aroF Δ aroG <i>ΔaroH::P_{J23119-rpsL-tac}(aroG^{S180F}-serA^{H344A/N364A}) P_{trc}-trpE^{S40F}DCBA</i>	Chen and Zeng (2017)
S028 λ	the parent strain of the strain S028, with the λ -red recombination system in the chromosome	Chen and Zeng (2017)
S028 λ C	S028 λ containing the plasmid pCas9	This work
WS001	S028 λ C Δ trpR:: <i>P_{tac}-tnaC-eGFP</i> (Trp sensor)	This work
WS002	WS001 Δ (<i>P_{J23119-rpsL-tac}-aroG^{S180F}</i>);(<i>P_{J23119-rpsL-CmR}-CmR</i>)	This work
WS003	WS002 Δ CmR:: <i>aroG^{WT}</i> without pCas	This work
WS004	WS002 Δ CmR:: <i>aroG^{S180F}</i> without pCas	This work
WS005	WS002 Δ CmR:: <i>aroG^{D6G-D7A}</i> without pCas	This work
S028GM1	S028 Δ aroG ^{S180F} :: <i>aroG^{D6G-D7A}</i>	This work
Top10	<i>F⁻ mcrA Δ(mrr-hsdRMS-mcrBC) ϕ80lacZΔM15 ΔlacX74 nupG recA1 araD139 Δ(ara-leu)7697 galE15 galK16 rpsL(StrR) endA1 λ</i>	Invitrogen
Plasmids		
pCas	expressing Cas9 protein and offering sgRNA for removing donor plasmid	Jiang et al. (2015)
pTargetF	plasmid for expressing sgRNA or with offering donor DNA, Spectinomycin resistance	Jiang et al. (2015)
pTagAmpR	plasmid for expressing sgRNA or with offering donor DNA, Ampicillin resistance	This work
pN20- <i>trpR</i>	pTagAmpR <i>trpR</i> -sgRNA ^a	This work
pN20- <i>aroG</i>	pTagAmpR <i>aroG</i> -sgRNA	This work
pCm- <i>aroG^{WT}</i>	pTagAmpR <i>CmR</i> -sgRNA Δ CmR:: <i>P_{J23119-rpsL-CmR}-aroG^{WT}</i>	This work
pCm- <i>aroG^{S180F}</i>	pTagAmpR <i>CmR</i> -sgRNA Δ CmR:: <i>P_{J23119-rpsL-CmR}-aroG^{S180F}</i>	This work
pCm- <i>aroG^{D6X-D7X}</i>	pTagAmpR <i>CmR</i> -sgRNA Δ CmR:: <i>P_{J23119-rpsL-CmR}-aroG^{D6X-D7X}</i>	This work
pCm- <i>aroG^{D6G-D7A}</i>	pTagAmpR <i>CmR</i> 202-sgRNA Δ CmR:: <i>P_{J23119-rpsL-tac}-aroG^{D6G-D7A}</i>	This work
pET22b(+)	pET vector	Novagen
pET- <i>aroG^{WT}</i>	pET22b inserted with <i>aroG^{WT}</i> gene	This work
pET- <i>aroG^{S180F}</i>	pET22b inserted with <i>aroG^{S180F}</i> gene	This work
pET- <i>aroG^{D6L-D7P}</i>	pET22b inserted with <i>aroG^{D6L-D7P}</i> gene	This work
pET- <i>aroG^{D6P-D7I}</i>	pET22b inserted with <i>aroG^{D6P-D7I}</i> gene	This work
pET- <i>aroG^{D6G-D7A}</i>	pET22b inserted with <i>aroG^{D6G-D7A}</i> gene	This work
pJLC	AmpR, PMB1, <i>P_{J23119-rpsL}-rpsL-CmR</i>	This work

^a *trpR*-sgRNA, sgRNA with an N20 sequence for targeting the *trpR* locus.

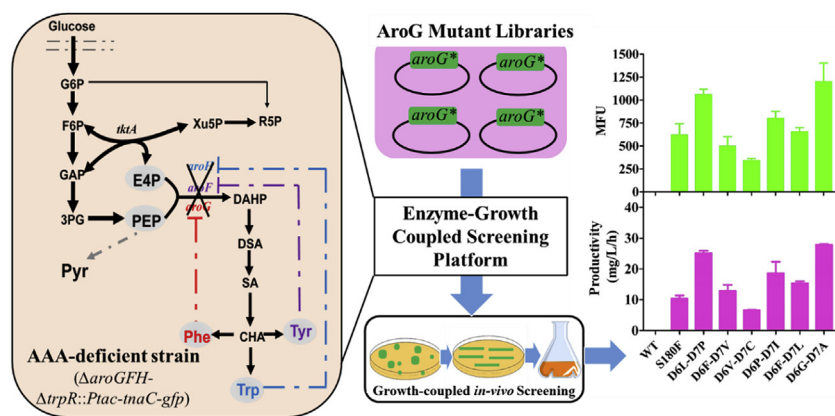


Fig. 2. Design and implementation of CGSS in screening of feedback-resistant AroG (AroG^{fbr}) enzyme variants. In *E. coli*, DAHP synthase (AroG, AroF, and AroH) is a key rate-limiting enzyme of the pathways for aromatic amino acids (AAAs) biosynthesis. An AAAs-auxotrophic strain (strain WS002) was constructed by disrupting the DAHP synthase and used as a platform for screening *aroG* gene variants, which were individually integrated into the chromosome of *E. coli* using the CRISPR/Cas9 system. In the presence of a high Phe concentration, only strains that express AroG^{fbr} with good resistance to Phe can produce enough AAAs and sustain cell growth. These strains were further characterized using the strength of the fluorescent signal (medium fluorescent unit, MFU) of Trp biosensor (P_{tac}-TnaC-eGFP) representing the productivity of Trp.

more than 24 h at 30 °C. Transformants were identified by colony PCR and DNA sequencing.

2.3. AroG variant library construction and screening

To demonstrate the efficiency of CGSS, we applied it to engineer Phe-resistant AroG. Two residues (Asp6 and Asp7) involved in the Phe-binding site of AroG (PDB ID: 1KFL) were chosen for saturated mutagenesis (NNK) in this study. The saturation mutagenesis was introduced with primers (Table S1) by amplifying the whole plasmid pCm-*aroG*^{WT} (Table 1) using the Phusion High-Fidelity PCR Master Mix (Thermo Scientific). After digestion of the template DNA with DpnI, the PCR products were then transformed into chemically competent *E. coli* Top10 cells, the reaction products were suspended in the SOB medium. After incubation at 37 °C for 1 h, all the cells were transferred into 10 ml fresh LB medium with 100 µg/ml ampicillin and cultivated for 8–10 h at 37 °C. The plasmids were extracted from these cultivations and eluted in water as AroG variant library: pCm-*aroG*^{D6X-D7X} (Fig. S1). More detail information for gene variant library construction is presented in the Supplementary Material. It is noted that mutations could be also introduced into linear donor DNA using PCR or oligonucleotides but they are showed to have a lower efficiency compared to the use of plasmid.

The AroG variant library was transferred into competent cells of WS002 (containing pCas). After 2 h incubation at 30 °C in the SOB medium, the transferred cells were washed with M9 medium (without any amino acids) three times and then spread onto M9 agar medium containing 25 mM Phe and 0.1 mM IPTG for screening. After incubation at 30 °C for more than 24 h, transformants with a bigger size and a stronger fluorescent signal were picked out and re-checked by streaking them on the same medium. Finally, the candidate strains were tested by cultivation in 5 ml fermentation medium II (FM-II) at 30 °C for 24 h. FM-II was nearly the same as reported previously (Gu et al., 2012), but contained 0.5 g/L instead of 5 g/L of MgSO₄·7H₂O and had 30 g/L initial glucose. Additionally, 12 g/L K₂HPO₄ (for buffering pH), 25 mM Phe and 0.1 mM IPTG were added. The mutants giving higher medium fluorescent (MFU) were selected for sequencing.

2.4. Method for measurement of fluorescent intensities

The mutants containing chromosomal Trp sensor with reporter eGFP protein cultured the LB medium were harvested by centrifugation and individually washed three times with the M9 medium to remove the LB medium. Afterward, each mutant was inoculated with the same amount of cells into 10 ml fresh M9 medium with 25 mM Phe in 50 mL conical tubes, and after a cultivation of 10 h cells were subjected to fluorescence analysis using flow cytometry. To this end, each culture was first washed three times using PBS buffer and diluted 100-fold and then eGFP fluorescence was monitored (MFI of ≥10,000 events) using a flow cytometer

(CytoFLEX, Beckman Coulter) at an excitation wavelength of 488 nm. All data were processed with the Beckman Flow software, and electronic gating was used to separate positive signals from instrument and water sample background. For fluorescent intensities, medium fluorescence unit (MFU) was calculated for each culture.

2.5. Protein expression and purification

For protein expression, the corresponding plasmids (see Section 2.1) were transformed into *E. coli* BL21, respectively. Protein expression and purification procedures were slightly modified from the one reported previously (Chen et al., 2018). The quantification of purified protein was performed according to Bradford's method (Bradford, 1976) using bovine serum albumin as standard and a prefabricated assay from Bio-Rad Laboratories (Hercules, CA).

2.6. Enzyme assay

In vitro enzyme kinetics of AroG^{fbr} mutants was performed as described in the literature with minor modification (Schoner and Herrmann, 1976). The enzyme activity was measured by monitoring the disappearance of PEP via absorbance at 232 nm, and the calculation of specific activity is according to the standard curve of PEP with the absorbance at 232 nm measured in the cuvette (Fig. S2). To investigate the effect of Phe on the activity of AroG variants, the activities were measured in the presence of different concentrations (from 0 to 40 mM) of Phe. The complete reaction mixture contained 10 mM Bis-tris propane (BTP, pH 7.0), 50 µM MnSO₄, 600 µM PEP, 500 µM E4P, and 25 µg purified enzyme in a total volume of 0.2 mL in the cuvette at 25 °C with or without inhibitor. The mixture without the substrates (PEP and E4P) and the substrates mixture (PEP and E4P) were equilibrated to the reaction temperature, separately, and the reaction was started by adding the substrates (PEP and E4P).

2.7. Fermentation conditions

For fed-batch fermentations in the bioreactor, the pre-culture and seed culture were performed under the same conditions as reported previously (Gu et al., 2012). Because the λ recombination system integrated into the strain *E. coli* DY330 can cause temperature-sensitive cell growth at 42 °C, it was removed from each strain derived from DY330 before it was used for fermentation. For this purpose, the procedure for removal of λ recombination system was performed according to the method reported by Chen and Zeng (2017). Fermentations were carried out in a highly instrumented and automated 4-parallel 1.5 L bioreactors system (DASGIP Parallel Bioreactor System, Jülich, Germany) with an initial working volume of 500 mL. If not indicated otherwise, the fermentation medium in bioreactor, feeding solution, and fermentation

conditions were the same as reported previously (Chen and Zeng, 2017).

2.8. Analytical methods

The quantification of glucose, 3-dehydroshikimate (DSA), and shikimate (SA) were done using HPLC as reported previously (Bommareddy et al., 2014; Luz et al., 2014). The determination of Trp was carried out by using a sensitive spectrophotometric method (Nagaraja et al., 2003).

3. Results and discussion

3.1. Proof-of-concept of the CGSS method

For proof-of-concept of the proposed CGSS method, we first constructed a screening strain which contains a Trp biosensor and lacks DAHP synthase activity for screening variants of DAHP synthase. To this end, deleting the *aroG^{S180F}* gene from our previously developed strain S028 λ (Chen and Zeng, 2017) made the strain auxotrophic for AAAs. Consequently, the growth of the mutant is linked to the DAHP synthase activity upon its re-introduction (Fig. 2). In principle, an engineered DAHP synthase with a higher activity should lead to a faster accumulation of Trp, which in turn stimulates the expression of a report gene regulated by the Trp biosensor (Fang et al., 2016).

The Trp biosensor is composed of *tnaC*, which encodes the leader sequence of the *tnaCAB* operon (Bischoff et al., 2014; Gong et al., 2001); the eGFP protein was fused to the upstream of *tnaC* (Fang et al., 2016). Specifically, the strain WS001 (Table 1) was first constructed based on the Trp producer S028 λ C (Table 1) after replacement of the *trpR* gene with the Trp biosensor using the CRISPR/Cas9 system. Then, we removed the sole DAHP synthase of the strain WS001 by replacing the *aroG^{S180F}* gene with the antibiotic resistance gene *Cm^R* which offers sgRNA targets for the CRISPR/Cas9 system in further genome-editing, generating the strain WS002 (Table 1, Fig. 2). More detailed information for strain construction is given in the Supplementary Material. As expected, the strain WS002 is not able to grow in the M9 medium without the addition of any of the aromatic amino acids Phe, Tyr, and Trp (data not shown). Then, we tested if the strain with a feedback resistant DAHP synthase will behave differently from the one having the wild-type DAHP synthase in terms of cell growth and expression of the report gene in a defined medium containing a high concentration of enzyme inhibitor. To this end, we introduced the wild-type *aroG* gene and the feedback-resistant gene *aroG^{S180F}* (Ger et al., 1994) into the chromosome of the strain WS002 at the locus of the *CmR* gene using the CRISPR/Cas9 technique with the plasmids pCm-*aroG^{WT}* and pCm-*aroG^{S180F}*, respectively. The recombinants were spread on M9-agar medium containing 25 mM Phe without Tyr and Trp. 0.1 mM IPTG was also supplemented into the medium for the following reasons. The first one is to repeal LacI-repression and thus to induce the expression of genes for the *trp* biosynthetic pathway since it is regulated by the LacI regulator. The other is to induce the expression of sgRNA which guides Cas9 to cut the donor plasmids from which the gene of interest can also be expressed.

The results showed that after introducing the *aroG^{S180F}* gene into the host, many recombinants with strong fluorescent signal grow up under the conditions mentioned above (Fig. 3). We have selected several colonies for further characterization. It turned out that these colonies all have the same mutation S180F. These recombinants were designated as WS004. No colony was observed when the wildtype *aroG* gene was integrated with the plasmid pCm-*aroG^{WT}* under the same conditions (Fig. 3). Presumably, the activity of the wild-type AroG is seriously inhibited by Phe and it could not support the growth of cells. It could be however also possible that the recombineering efficiency is too low. To eliminate the latter possibility, the recombinants with the *aroG^{WT}* gene were also grown on LB-agar medium with IPTG. From the complex medium, we obtained many colonies (Fig. 3) which were positively confirmed by colony PCR and designated as WS003. The strain WS003 and WS004 were re-checked on M9-agar medium with and without

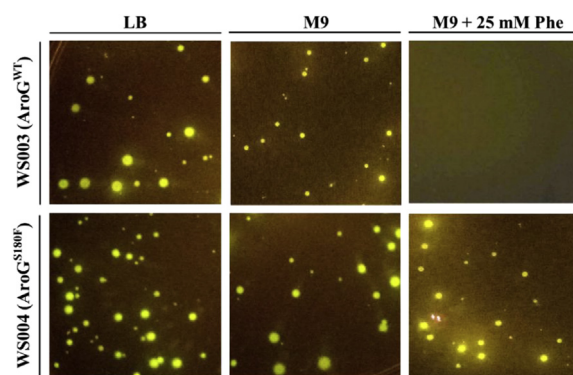


Fig. 3. Comparison of the growth and fluorescence of the two strains WS003 and WS004 generated by introducing *aroG^{WT}* and *aroG^{S180F}* into the chromosome of the strain WS002, respectively, under different growth conditions. The fluorescent signal of colonies grown on the agar plate was detected under UV light. Left, complex medium (LB-agar); middle, M9-agar (without any amino acids); right, M9-agar with 25 mM Phe.

25 mM Phe (Fig. 3). The strain WS003 was found to grow on the medium without Phe but no growth was observed on the medium with Phe (Fig. 3). As expected, the growth of the strain WS004 did not show a notable difference on the media with or without Phe. These results suggested that CGSS is an approach useful to facilitate engineering of enzymes with desired performance such as higher activity and higher inhibitor tolerance. It is used in the following to obtain AroG variants with further improved tolerance against Phe.

3.2. CGSS for screening Phe-resistant AroG

To demonstrate the usefulness of the CGSS method established above for obtaining more resistant AroG enzyme variants, a mutation library of AroG was first generated.

For this purpose, a semi-rational strategy was adopted which makes use of information from the crystal structure of AroG complexed with its inhibitor Phe (PDB: 1KFL) (Fig. 4a). The residues D6 and D7 involved in the binding of Phe were selected as targets to perform saturation mutagenesis. They were then screened with the CGSS. As shown in Fig. 4b, for the AroG^{D6X-D7X} variants, there were around 100 colonies with different size and different strength of fluorescent signal obtained on the previously mentioned screening medium after grown for about 30 h.

After the first round screening, 30 colonies of the AroG^{D6X-D7X} variants, which have relatively bigger size and higher fluorescence signal, were selected and re-checked on the screening medium. After confirmation of the phenotypes, the mutated *aroG* genes from 20 candidates were isolated for sequencing. The sequencing results showed that there are only 6 different AroG variants among the 20 candidates (Table 2). They are AroG^{D6G-D7A}, AroG^{D6L-D7P}, AroG^{D6P-D7I}, AroG^{D6F-D7V}, AroG^{D6V-D7C}, and AroG^{D6F-D7L}, with the number of occurring being 7, 6, 4, 1, 1, and 1, respectively (Table 2). We then carried out fermentations in FM-II medium in 50 mL conical tubes with the strains carrying these 6

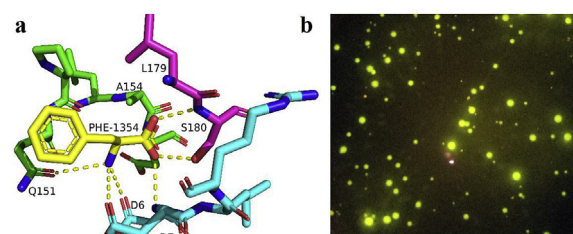


Fig. 4. (a) Key residues involved in the Phe binding sites of AroG from *E. coli*. (b) The fluorescence induction for AroG^{D6X-D7X} variants on reduced M9-agar (without Tyr and Trp) with the addition of 25 mM Phe and 0.1 mM IPTG.

Table 2

Comparison of fermentation results with *E. coli* strains containing the AroG^{WT}, AroG^{S180F}, and AroG^{fbr} variants grown on FM-II with 25 mM Phe.

ID	Mutated Residues	Number of occurrence ^a	DCW (g/L)	Trp (g/L)
N1	WT	–	0.786 ± 0.145	N. D.
N2	S180F	–	0.714 ± 0.045	0.248 ± 0.025
N3	D6L-D7P	6	0.983 ± 0.118	0.604 ± 0.018
N4	D6F-D7V	1	0.987 ± 0.007	0.309 ± 0.047
N5	D6V-D7C	1	1.071 ± 0.094	0.157 ± 0.004
N6	D6P-D7I	4	1.001 ± 0.009	0.448 ± 0.089
N7	D6F-D7L	1	1.015 ± 0.156	0.368 ± 0.017
N8	D6G-D7A	7	1.102 ± 0.105	0.670 ± 0.005

^a "Number of variants" refers to all the 20 candidates examined; The fermentations were performed with a single clone for each AroG-variant; N. D., not detected. The average value ± standard deviation is based on three independent experiments.

recombinants and compared them with the wild-type strain WS003 and the strain WS004 has AroG^{S180F} variant. As shown in Table 2, 19 of the mutants had a higher Trp production compared to the strain WS004. It suggested that the efficiency for generating positive mutants is remarkable. Moreover, it was found that the strains carrying the variants AroG^{D6G-D7A}, AroG^{D6L-D7P}, or AroG^{D6P-D7I}, which had the higher frequency of occurrence in the 20 candidates (Table 2), had also higher Trp productivity (Fig. 2). Especially the first two mutants had much higher productivity than that of the reference strain (AroG^{S180F}). In addition, we investigated the relationship between the Trp productivity and the strength of fluorescence signal. The strain which has a stronger fluorescence signal also has a higher Trp productivity (Fig. 2). These results suggest that the variants AroG^{D6G-D7A}, AroG^{D6L-D7P}, and AroG^{D6P-D7I} have a higher catalytic efficiency than the variant AroG^{S180F} under the test conditions. To provide more direct evidence, enzyme assay was done with the purified protein of these variants.

3.3. Characterization of selected AroG^{fbr} variants *in vitro*

To examine if the higher Trp productivity and stronger fluorescence signal observed in the strains is due to improved Phe-tolerance of the corresponding AroG variants we investigated the inhibition behavior of the variants AroG^{D6G-D7A}, AroG^{D6L-D7P}, and AroG^{D6P-D7I}.

As shown in Fig. 5, all the variants are significantly less sensitive to the inhibitor Phe, while the wild-type AroG is extremely sensitive to it. In the presence of 0.5 mM Phe, the wild-type AroG almost completely lost its activity, whereas all the variants remained more than 80% of their activities under the same conditions. Compared to AroG^{S180F}, all of the three variants AroG^{D6G-D7A}, AroG^{D6L-D7P}, and AroG^{D6P-D7I} have weaker sensitivities to Phe when the concentration of Phe was higher than 10 mM (Fig. 5b). They also had higher specific activities in the presence

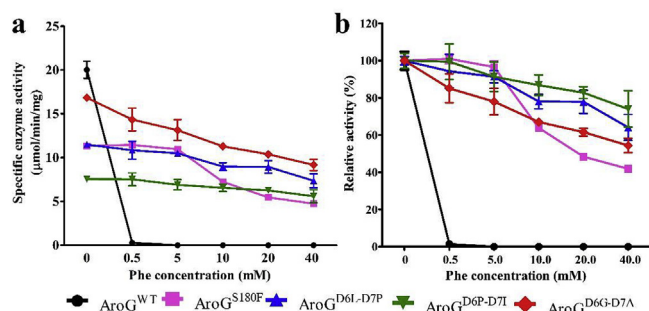


Fig. 5. Effect of Phe on the activities of the enzyme AroG^{WT} and its variants AroG^{S180F}, AroG^{D6L-D7P}, AroG^{D6P-D7I}, and AroG^{D6G-D7A}. (a) Specific activities; (b) Relative activities. Results were derived from three independent experiments.

of more than 20 mM Phe (Fig. 5a). These results explain why the strains with these three variants performed better than the positive control in terms of the Trp production when they were cultivated in medium containing a very high Phe concentration (Table 2). As shown in Fig. 5a, the specific activities are significantly different between these three variants generated by the substitution of the same residues D6 and D7. Among them, AroG^{D6G-D7A} variant has the highest specific activity, which is nearly twice as high as that of the variant AroG^{D6P-D7I} regardless of the Phe concentration. It's also remarkably higher than that of the positive control AroG^{S180F}.

3.4. Improvement of the chorismate pathway and trp biosynthesis

To explore the impact of our best variant AroG^{D6G-D7A} on strain development for the biosynthesis of aromatic amino acids, the variant AroG^{S180F} in our previously constructed Trp-producing strain S028 was substituted with AroG^{D6G-D7A}, generating the strain S028GM1 (see Supplementary Material). The capacity of Trp production of S028GM1 strain was compared to that of the reference strain S028 by carrying out simple fed-batch fermentations in bioreactors.

As shown in Fig. 6a and c, the strain S028GM1 produced a significant higher amount of Trp than the reference strain after the lag phase (about 10 h) till the end of the fermentation. At the end of the fermentation (37 h), the strain S028GM1 produced 24.03 ± 1.02 g/L, which is 38.50% higher than that (17.35 ± 1.16 g/L) of the strain S028 (Fig. 6c). The concentrations of glucose were controlled at nearly the same level for both strains during the whole fermentation. It was found that the strain S028GM1 had a slightly faster growth rate (0.211 h⁻¹) than the strain S028 (0.184 h⁻¹, Fig. 6a) at the beginning of the exponential growth phase. The enhanced DAHP synthase activity has obviously contributed to the higher growth rate to a certain extent. A higher biomass formation rate could reasonably result in a higher productivity for the strain S028GM1 (Fig. 6d). However, its higher Trp production can be considered to be mainly due to the enhanced DAHP synthase activity directly, because the specific Trp formation rate of the strain S028GM1 was remarkably higher than that of the strain S028 (Fig. 6e) during the whole fermentation. In addition, during the fermentation, the strain S028GM1 accumulated higher amounts of the intermediates SA and DSA (Fig. 6f) of the chorismate pathway than the reference strain S028. Both of the intermediates accumulated by the strain S028GM1 are about 36% higher than those produced by S028 at the end of fermentation. It suggests that more metabolic flux was redirected into the chorismate pathway in the strain S028GM1 than in the reference strain due to the difference between the variants AroG^{D6G-D7A} and AroG^{S180F}. These results clearly showed that the variant AroG^{D6G-D7A} is more efficient for the chorismate pathway towards bio-production of aromatic amino acids and their derivatives.

4. Conclusion

Protein engineering in the context of metabolic engineering is of fundamental importance for developing efficient bioprocesses. While impressive progress has been made in the design of enzyme variant library, the availability of reliable and highly effective screening methods becomes more and more important. In this work, a novel approach of integrating CRISPR/Cas9-facilitated engineering with growth-coupled and sensor-guided *in vivo* screening (CGSS) has been developed to facilitate protein engineering. This approach is successfully demonstrated for the engineering and screening of 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase (AroG) to discover AroG variants which are more resistant to Phe and thus more suitable for the biosynthesis of aromatic amino acids like Trp. With two selected mutation points based on structural information, new variants (AroG^{D6G-D7A}, AroG^{D6L-D7P}, and AroG^{D6P-D7I}) were revealed to be more resistant to Phe than the Phe-resistant variant AroG^{S180F} reported in the literature. The replacement of AroG^{S180F} with AroG^{D6G-D7A} in a previously engineered Trp producing

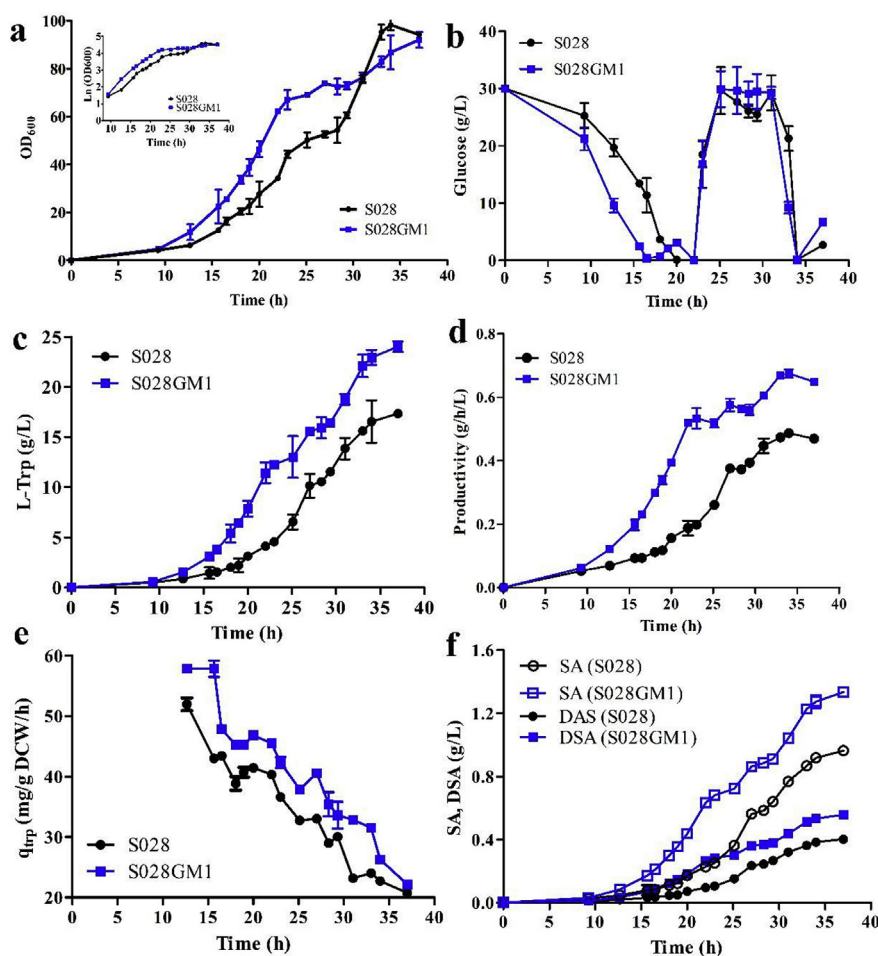


Fig. 6. Fed-batch fermentation results of the strains S028 (black and circle) and S028GM1 (blue and square). (a) Cell growth; (b) Glucose concentration; (c) Trp production; (d) Overall productivity, (e) Formation rate of Trp (q_{Trp}), and (f) Accumulation of the intermediates shikimate (SA, open circle or square) and dehydroshikimate (DSA, solid circle or square). All results are based on two independent fermentations.

E. coli strain (S028) remarkably increased the Trp production by 38.5% in a simple fed-batch fermentation. Since CGSS is based on an integration of the AroG-encoding gene variants into the chromosome, it can also be simultaneously used to optimize the expression level of the engineered enzyme in the strain, i.e. by constructing the corresponding gene using different promoters and/or ribosome binding sites. It is worthwhile to mention that the high efficiency of CRISPR/Cas9 technology allows multiplex genome editing. This could help to address a wide range of targets that require a simultaneous modulation of multiple genes, e.g. for the synthesis of metabolites like Trp requiring multiple precursors.

Compliance with ethical standards

Conflict of interest

The authors declare that they have no conflict of interest.

Ethical statement

The article does not contain any studies with human participants or animals performed by any of the authors.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.mec.2019.e00094>.

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