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Engineering *Escherichia coli* to sense acidic amino acids by introduction of a chimeric two-component system

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Abstract−In an attempt to create an acidic amino acid-sensing *Escherichia coli*, a chimeric sensor kinase (SK)-based biosensor was constructed using *Pseudomonas putida* AauS. AauS is a sensor kinase that ultimately controls expression of the *aau* gene through its cognate response regulator AauR, and is found only in *P. putida* KT2440. The AauZ chimera SK was constructed by integration of the sensing domain of AauS with the catalytic domain of EnvZ to control the expression of the *ompC* gene in response to acidic amino acids. Real-time quantitative PCR and GFP fluorescence studies showed increased *ompC* gene expression and GFP fluorescence as the concentration of acidic amino acids increased. These data suggest that AauS-based recombinant *E. coli* can be used as a bacterial biosensor of acidic amino acids. By employing the chimeric SK strategy, various bacteria biosensors for use in the development of biochemical-producing recombinant microorganisms can be constructed.

Keywords: Acidic Amino Acids, Chimera Two-component System, Green Fluorescent Protein, *Escherichia coli*

INTRODUCTION

Amino acids have been used for industrial purposes and as food additives, since the commercialization of glutamate in 1909. Glutamate, lysine, tryptophan, methionine and threonine are the most commonly produced amino acids [1]. Glutamate has been used as a flavor enhancer in the food industry, while lysine is mainly used as an additive to animal feed [2]. Typically, about 1.5 million tons of glutamate and 0.8 million tons of lysine are produced yearly. Considering the industrial importance of amino acids, various studies have been done to improve amino acid productivity during the last 50 years [3]. Though intensive research has been focused on the metabolism of amino acid producing bacteria, the carbon metabolic network and regulation mechanism have not yet been clearly revealed. Therefore, the traditional 'trial-and-error' strategy is still being used for the development of more efficient amino acid producers, and a high-throughput screening method is required to allow more efficient identification of amino acid producers. An amino acid bacteria sensor can be considered as one of the promising candidates for a high-throughput screening method.

Bacteria can sense environmental conditions via various apparatus, of which two-component systems (TCS) are one of the most efficient environmental sensors. TCS consists of a sensor kinase (SK) and a response regulator (RR) as the basic elements [4,5]. When the membrane-bound SK senses an environmental stimulus, it activates the corresponding RR through phosphorylation, which induces the transcription of related genes [6]. It has been reported that the signal characteristics of TCSs can be engineered by constructing chimeric SKs. The SK consists of an N-terminal sensor domain and a C-terminal catalytic domain, and chimera SKs with the desired characteristics can be constructed by combination of the domains from different SKs [7].

Chimera SKs have proven to be a powerful tool for engineering TCSs with new and novel properties. The sensor domain of the aspartate sensing SK, Tar, and the catalytic domain of the osmolarity sensing SK, EnvZ, were integrated to construct the SK Taz1 chimera, which induces expression of *ompC* gene in response to aspartate [7]. The light sensing phytochrome Cph1 from *Cyanobacterium* was later fused to EnvZ to create the Cph8 chimera, which showed strong response to light [8]. Similar to the above strategies, MzrA-EnvZ chimera was constructed to study the dynamic behavior of the EnvZ/OmpR regulon [9]. Because the EnvZ/OmpR is one of the best characterized TCS in *Escherichia coli*, EnvZ has been used for the construction of novel signaling pathways.

The genome of *Pseudomonas putida* KT2440 encodes more than 130 SKs and RRs that make up at least 50 different two-component systems and most of its putative regulatory systems. *P. putida* KT2440 metabolizes a wide range of carbon and nitrogen sources, including many amino acids. During the growth of *P. putida* KT2440 on glutamate as a sole source of carbon along with nitrogen, a number of proteins are upregulated, including a periplasmic glutaminase/asparaginase and an ATP-binding cassette-type transporter. It was reported that the TCS encoded by PP1067-PP1066 (*aauS-aauR*) is required for the efficient growth of *P. putida* KT2440 on acidic amino acids (Glutamate and aspatate) and their amides (Glutamine and asparagine) [10].

In this study, the sensing domain of AauS was fused with the

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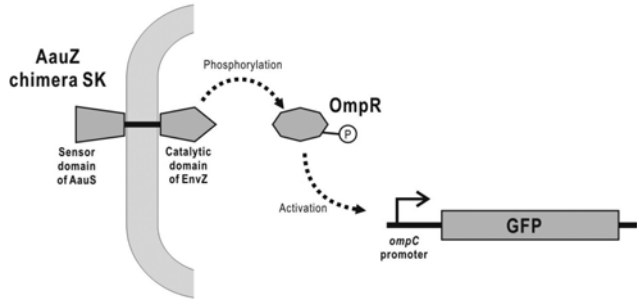

Fig. 1. Acidic amino acids sensing recombinant *Escherichia coli*.

catalytic domain of EnvZ to construct chimera AauZ SK, which was subsequently introduced into *E. coli* to construct an acidic amino acid-sensing recombinant *E. coli*, which does not have acidic amino acid sensing TCS in nature (Fig. 1). The dynamic behavior of the AauZ chimera in response to acidic amino acids was monitored by quantitative real-time PCR (qRT-PCR) and green fluorescent protein (GFP) [11].

MATERIALS AND METHODS

1. Bacterial Strains and Culture Conditions

E. coli BL21 (DE3) was used as the host strain for recombinant DNA manipulation. The bacterial strains, plasmids and primers used in this study are listed in Table 1. Unless otherwise stated, all *E. coli* strains were grown aerobically in Luria-Bertani (LB) broth (10 g l^{-1} bacto-tryptone, 5 g l^{-1} bacto-yeast extract and 5 g l^{-1} NaCl) and in M9 minimal salts medium (Sigma) using glucose (0.4%) as a carbon source with supplements of 2 mM $MgSO_4$, 0.1 mM $CaCl_2$ and 1% thiamine HCl. Media were supplemented with antibiotics (25 μg ml^{-1} chloramphenicol and/or 100 μg ml^{-1} ampicillin), and the strains were cultured at 30 °C under vigorous shaking.

2. Construction of Plasmids Containing AauZ and Reporter Plasmid

The plasmids pAauZ1 and pOGFP1 were constructed for the expression of chimera AauZ SK and the GFP fluorescence reporter protein, respectively. The chimera *aauZ* gene was constructed by overlap polymerase chain reaction (PCR). The 360-amino acid sensor domain of AauS was amplified from *P. putida* KT2440 genomic DNA, and the 228-amino acid catalytic domain of EnvZ was amplified from *E. coli* BL21 genomic DNA. The two PCR products were integrated by overlap PCR and cloned into a low copy number pACYCDuet-1 plasmid to construct pAauZ1 using the AauS_F_*Bam*HI and EnvZ_R_*Hin*dIII restriction sites. The *aauZ* gene was placed under the control of the T7 promoter, stimulated by the addition of isopropyl-beta-D-thiogalactopyranoside (IPTG).

The genomic region containing 250 bp of the *ompC* promoter was amplified from *E. coli* XL1-Blue genomic DNA with oligonucleotides OmpC_F_*Eco*RI and OmpC_R_*Bam*HI (Table 2). Oligonucleotides GFPm_F_*Bam*HI and GFPm_R_*SalI* (Table 2) were

Table 1. List of bacterial strains and plasmids used in this study

Strain/plasmid	Relevant genotype and/or property	Source
***Escherichia coli* strains**		
TOP10	*F- mcrA Δ (mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 nupG recA1 araD139 Δ (ara-leu)7697 galE15 galK16 rpsL(Str^R) endA1 λ$^-$*	Laboratory stock
BL21(DE3)	*F$^-$ ompT gal dcm lon hsdS_B(r_B^- m_B^-) λ(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5])*	Laboratory stock
Plasmids		
pUC19	Amp^R	New England Biolabs[a]
pOGFP1	pUC19 containing the *ompC* promoter and *gfp* gene, Amp^R	This work
pACYCDuet-1	Cm^R	Novagen
pAauZ1	pACYCDuet-1 derivative, containing the chimeric *aauS-envZ* gene, Cm^R	This work

[a]New England Biolabs, Beverly, MA, U.S.A.

Table 2. Oligonucleotide primers used in this study[a]

Name	Sequence
AauS_F_*BamHI*	5′-CG**GGATCC**ATGAGACATTCATTGCCCTAC-3′
AauS_R	5′-AGATCCACCACCGCCAGAGCCACCGCCACCCAGCGTGGAGATTTCGTA-3′
EnvZ_F	5′-GGCGGTGGTGGATCTGGTGGCGGCGGTTCTATGGCGGCTGGTGTTAAG-3′
EnvZ_R_*HindIII*	5′-CCC**AAGCTT**TTATTACCCTTCTTTTGTCGT-3′
OmpC_F_*Eco*RI	5′-**GAATTC**TTGAATTATTATTGCTTGATG-3′
OmpC_R_*Bam*HI	5′-**GGATCC**GTTATTAACCCTCTGTTATAT-3′
GFPm_F_*Bam*HI	5′-**GGATCC**CATATGCAGTCTAAAGGAGAA-3′
GFPm_R_*Sal*I	5′-**GTCGAC**CTCGAGTTATTAATGGTGAT-3′
OmpC_RT_Fwd	5′-CTTCAAAGGTGAAACTCAGGTTACTG-3′
OmpC_RT_Rev	5′-GTTGCCCTGGATCTGATATTCC-3′

[a]Restriction enzyme sites are shown in bold

used to amplify the *gfp* gene from GFP-mut3.1, which was previously cloned in the pET expression system [12]. The *ompC* promoter was then ligated with the *gfp* gene, after which the product was cloned into pUC19 to construct pOGFP1.

3. Monitoring of *ompC* Gene Expression by qRT-PCR

The transcriptional expression of the *ompC* gene in *E. coli* BL21 (DE3) harboring pAauZ1 in response to the presence of acidic amino acids was measured by qRT-PCR. A single colony of the recombinant *E. coli* strain was grown overnight in LB medium at 37 °C. It was then diluted 100-fold in fresh M9 medium (supplemented with 25 μg ml^{-1} chloramphenicol) and incubated at 37 °C in an orbital shaker at 250 rpm until the optical density at 600 nm (OD_{600}) reached 0.5. IPTG (10 mM) was then added to the culture, and the cells were grown aerobically for an additional 4.5 h at 30 °C in the presence of varying concentrations of acidic amino acids. After 4.5 h, the cells were harvested by centrifugation for total RNA preparation using the RNeasy Mini kit (Qiagen), followed by DNase treatment. Reverse transcription was performed with a cDNA synthesis kit (Applied Biosystems) using a random hexamer primer mix, according to the manufacturer's instructions. Specific primers were designed with OLIGO software (version 5.0; Molecular Biology Insights, Cascade, CO) to quantify the expression of the *ompC* gene and 16sRNA (Table 2). Samples for which the RT step was omitted were used as negative controls to check that the extracted RNA was not contaminated with DNA. qRT-PCR reactions were performed on the Mini-Opticon detection system using SYBR Green PCR Master Mix, as recommended by the manufacturer. Each qRT-PCR experiment was performed in triplicate for biological samples using separate cultures grown under identical conditions (n=3), and quantification was performed automatically by the Mini-Opticon software using 16sRNA as an internal control [13,14].

4. Fluorescence-based Expression Monitoring

A single colony of *E. coli* BL21 (DE3) strain harboring the pAauZ1 and pOGFP1 plasmids was grown overnight at 37 °C in LB medium. The overnight culture was diluted 100-fold in minimal (M9) medium supplemented with 100 μg ml^{-1} ampicillin and 25 μg ml^{-1} chloramphenicol, and incubated at 37 °C until the OD_{600} reached 0.5. Expression of the chimera AauZ SK was then induced with 10 μM IPTG, and the strain was incubated for an additional 16 h at 30 °C.

Various concentrations of glutamate, aspartate and glutamine were added to the culture medium. Cell growth was monitored by measuring the optical density at 600 nm with a spectrophotometer (Shimadzu, Japan). The fluorescence of GFP was measured using an RF-5301PC spectrophotometer (Shimadzu, Japan) with an excitation wavelength of 485/10 nm and an emission wavelength of 515/10 nm. The specific fluorescence intensity (SFI) was calculated as the raw fluorescence intensity expressed in relative fluorescence units divided by the optical density at 600 nm measured at each time point. A minimum of triplicate measurements were obtained for each sample.

5. Principal Component Analysis (PCA)

The input for analysis was a 4 X 3 matrix where each row corresponded to the three amino acids and each column corresponded to the particular induction concentrations (0.1, 1.0, 5.0 and 10 mM) used in this study. The output of the PCA was a 4 X 3 matrix. In this form, each amino acid was represented as a point in three dimensions, where each of the axes was a principal component. Each principal component was a linear combination of the three different concentrations of metabolites used. The contributions of each concentration were PC1 and PC2, where each metabolite corresponded to the chimera AauZ/OmpR TCS. PC1 and PC2 together captured 100% of the variance in the original dataset.

RESULTS AND DISCUSSION

1. Construction of Chimera TCS and Reporter Systems

Since *E. coli* does not have an amino acid-sensing TCS, a synthetic TCS was introduced to construct an *E. coli*-based acidic amino acid-sensing bacterial biosensor. The σ^{54}-dependent TCS, which controls the uptake and metabolism of acidic amino acids, was previously reported in *P. putida* KT2440 [10]. In the present study, the sensor domain of AauS was integrated with the catalytic domain of EnvZ to construct the chimeric SK AauZ, which activates OmpR to induce the expression of the *ompC* gene. Because intensive expression of membrane-bound AauZ chimera SK may cause destabilization of the cell membrane or reduction in cellular activity, the *aauZ* gene was cloned into the very low copy number vector, pACYCDuet-1. To monitor the expression of the *ompC* gene in response to the presence of extracellular acidic amino acids via induction by the AauZ/OmpR TCS, a fluorescence-based reporter plasmid was constructed. The *gfp* gene was cloned downstream of the *ompC* promoter to construct the pOGFP1 plasmid. Therefore, GFP was expressed when the AauZ chimera sensed extracellular acidic amino acids.

2. Monitoring of *ompC* Gene Expression by qRT-PCR

The dynamic expression characteristics of the *ompC* gene, which was induced by the newly constructed AauZ/OmpR chimera TCS in response to acidic amino acids, was monitored via qRT-PCR. The recombinant *E. coli* strain containing the chimera AauZ SK was cultured in minimal M9 media supplemented with varying concentrations of acidic amino acids, and the expression of the *ompC*

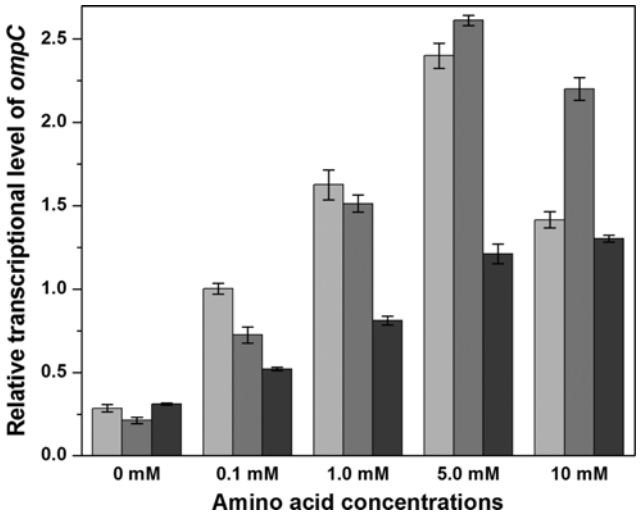

Fig. 2. Transcriptional level of *ompC* gene in response to various amino acids. Glutamic acid (light grey), aspartic acid (grey) and glutamine (dark grey).

gene was analyzed (Fig. 2).

Expression of the *ompC* gene was induced by the addition of 0.1 mM glutamate, and the expression level was observed to increase continuously, while the glutamate concentration increased to 5 mM. When 10 mM of glutamate was added to the media, the level of *ompC* expression decreased to that obtained with 1 mM glutamate (Fig. 2). A similar *ompC* gene expression profile was obtained for aspartate, which increased until 5 mM, then decreased with further increases in concentration. When glutamine was added to the media, only small changes in the *ompC* expression profile were observed. Unlike glutamate and aspartate, the *ompC* expression level increased continuously until 10 mM of glutamine was added, while the expression levels were lower than those obtained with the same concentrations of glutamate and aspartate. These results clearly indicated that the level of *ompC* gene transcription can be regulated by the presence of amino acids via the newly constructed chimeric AauZ/OmpR TCS.

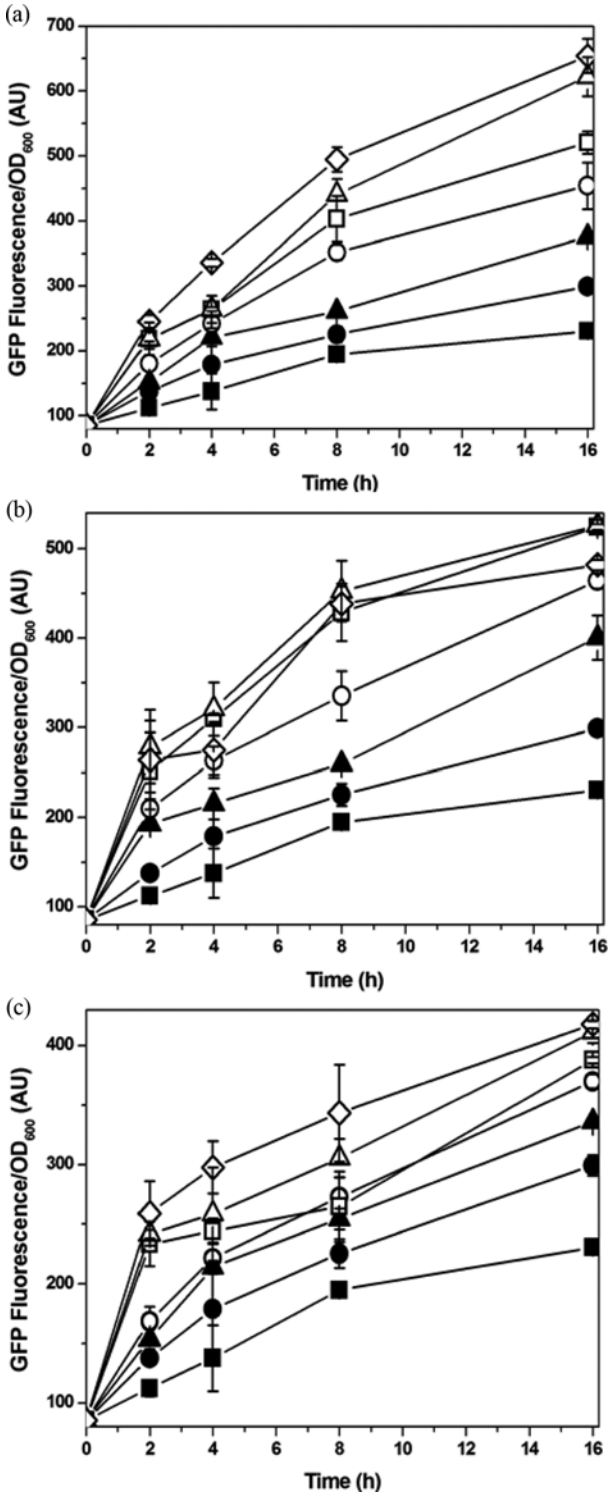

Fig. 3. Time course of GFP fluorescence in *E. coli* (pAauZ1 and pOGFP1) after induction with varying concentrations of glutamic acid (a), aspartic acid (b) and glutamine (c). Control (closed square); 0 mM (closed circle); 0.05 mM (closed triangle); 0.1 mM (open circle); 1.0 mM (open square); 5.0 mM (open triangle); 10 mM (open diamond).

3. Fluorescence-based Monitoring of *ompC* Gene Expression

Because *ompC* gene expression was shown to be controlled by the AauZ/OmpR TCS chimera in response to acidic amino acids, an AauZ/OmpR-based amino acid bacterial biosensor was constructed. The *gfp* gene was cloned downstream of the *ompC* gene promoter, and GFP was expressed via the AauZ/OmpR TCS chimera. An *E. coli* strain harboring pACYCDuet-1 and pUC19 instead of pAauZ1 and pOGFP1 was used as the control strain.

Generally, higher levels of fluorescence were observed as the concentration of amino acids increased (Fig. 3). When glutamate was added to the media, higher levels of fluorescence were achieved by increasing the concentration, with maximum fluorescence obtained at the glutamate concentration of 10 mM. Similar expression profiles were obtained by the addition of aspartate, but the maximum fluorescence was observed at an aspartate concentration of 5 mM. When glutamine was added to the media, the relative fluorescence also increased as glutamine concentration increased. These results suggested that the AauZ/OmpR-based amino acid sensing apparatus can be applied as a bacterial biosensor for acidic amino acids.

Without the addition of amino acids, a very small increase of fluorescence was observed (Fig. 3). This may be due to the basal level of expression of *gfp*, which was cloned into the high copy number plasmid pUC19. Cloning the *ompC* promoter-*gfp* reporter system into a mid or low copy number plasmid could be considered as a solution for the problem of basal level expression.

4. Specificity Analysis of the Chimera TCS

To evaluate the substrate-specific expression characteristics of the constructed AauZ/OmpR TCS-based biosensor, the relative fluorescence data obtained under glutamate, aspartate and glutamine stimulation were analyzed. Four different concentrations (0.1, 1.0, 5.0 and 10 mM) of the amino acids were supplemented into the culture media, and the fluorescence was measured. Generally, glutamate and aspartate provided stronger fluorescence, while less strong fluorescence was obtained for glutamine (Fig. 4(a)).

Principal component analysis (PCA) was applied to the fluorescence data to analyze the effects of the amino acids on the activity of the chimera TCS (Fig. 4(b)). PCA can be applied to a dataset for multivariate data analysis by visualizing the diversity and clustering of patterns [15,16]. It reduces the dimensionality of the data and, as a result, a new axis depicts most of the information from the existing data set. We used PCA to capture the differences in

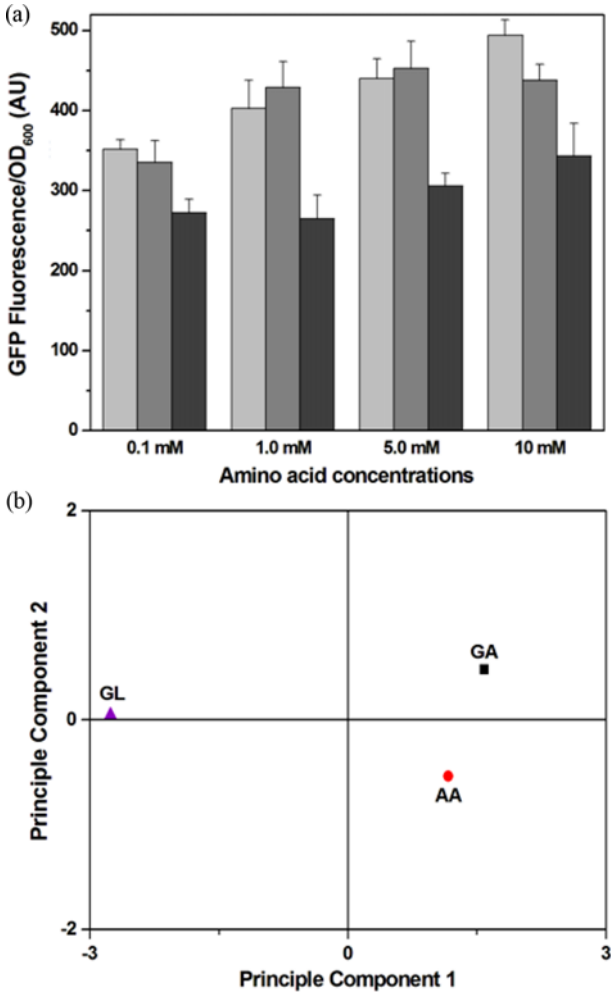

Fig. 4. (a) GFP fluorescence in *E. coli* (pAauSZ1 and pOGFP1) with varying concentrations of amino acids. Glutamic acid (light grey), aspartic acid (grey) and glutamine (dark grey). (b) PCA result obtained with aspartic acid (AA), glutamic acid (GA) and glutamine (GL).

the patterns exhibited by the engineered bacteria in response to different acidic amino acids at various concentrations. The input was a 4 X 3 matrix, where rows corresponded to the different metabolites used in this study and columns corresponded to varying concentrations (0.1, 1.0, 5.0 and 10 mM) of the three different amino acids. The acidic amino acids, glutamate and aspartate, were clustered together, while glutamine alone was located far away (Fig. 4(b)). This result suggested that the newly constructed AauZ/OmpR-based bacterial biosensor has a unique expression pattern with respect to glutamate and aspartate (Fig. 4(b)).

CONCLUSION

Chimera AauZ SK was constructed to sense environmental acidic amino acids by integration of AauS from *P. putida* KT2440 and EnvZ from *E. coli*. AauS/AauR from *P. putida* KT2440 is the first TCS shown to participate in the utilization of acidic amino acids. Despite the very low sequence identity between the sensor domains of AauS and EnvZ, the acidic amino acid-sensing domain of AauS was successfully recombined with the catalytic domain of EnvZ. The constructed chimera, AauZ SK, recognized the same effecter molecules as the original AauS SK [10]. This result underlines the efficiency of the chimera protein strategy for the construction of novel TCS with desired characteristics.

Further studies are required to improve the sensing ability of the AauZ chimera protein, which can aid in the quantification of amino acids, and to use the AauZ/OmpR TCS-based system as an amino acid-sensing bacterial biosensor. Herein, we described the design, construction and function of AauZ chimera protein in order to understand the molecular mechanisms of the AauZ/OmpR TCS. This newly constructed chimera system may also be used for the high-throughput screening of amino acid-producing microorganisms.

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