Unraveling the kinetic diversity of microbial 3-dehydroquinate dehydratases of shikimate pathway

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Abstract

3-Dehydroquinate dehydratase (DHQase) catalyzes the conversion of 3-dehydroquinic acid to 3-dehydroshikimic acid of the shikimate pathway. In this study, 3180 prokaryotic genomes were examined and 459 DHQase sequences were retrieved. Based on sequence analysis and their original hosts, 38 DHQase genes were selected for chemical synthesis. The selected DHQases were translated into new DNA sequences according to the genetic codon usage bias by both *Escherichia coli* and *Corynebacterium glutamicum*. The new DNA sequences were customized for synthetic biological applications by adding Biobrick adapters at both ends and by removal of any related restriction endonuclease sites. The customized DHQase genes were successfully expressed in *E. coli*, and functional DHQases were obtained. Kinetic parameters of $K_m$, $k_{cat}$, and $V_{max}$ of DHQases were determined with a newly established high-throughput method for DHQase activity assay. Results showed that DHQases possessed broad strength of substrate affinities and catalytic capacities. In addition to the DHQase kinetic diversities, this study generated a DHQase library with known catalytic constants that could be applied to design artificial modules of shikimate pathway for metabolic engineering and synthetic biology.

Keywords: 3-Dehydroquinate dehydratase (DHQase); Kinetic constants; Shikimate pathway; Biobrick; Synthetic biology

Introduction

Shikimate pathway widely exists in microbes and plants, but not animals. This pathway is involved in the synthesis of aromatic amino acids, vitamins, as well as lignin (Herrmann and Weaver 1999; Vanholme et al. 2012). The pathway consists of seven catalytic steps, condensing erythrose-4-phosphate (E4P) and phosphoenolpyruvate (PEP) finally into chorismate. 3-Dehydroquinate dehydratase (DHQase, EC 4.2.1.10) catalyzes the third step, i.e., reversible transformation of DHQ to form 3-dehydroshikimic acid. DHQases belong to the family of lyases, and cleave carbon-oxygen bonds. So far as known, DHQases involve in not only shikimate pathway but also other metabolic processes such as the quinate pathway for synthesis of 4-hydroxybenzoic acid (Giles et al. 1985; Giles et al. 1991). According to their origins and catalytic features, DHQases are classified into either type I or type II. Type I DHQases are heat-labile dimeric (Roszak et al. 2002), and mainly occur in plants and fungi. Type II DHQases are heat-stable dodecameric (Roszak et al. 2002) and widely occur in bacteria for shikimate pathway or in fungi for quinate catabolism (Giles et al. 1991).

Many investigations of DHQases have been focused on their structures and catalytic mechanisms (Blomberg et al. 2009; Bottomley et al. 1996; Devi et al. 2013; Lee et al. 2002; Pan et al. 2012; Roszak et al. 2002), or on structure-based design of inhibitors to DHQase activity (Blanco et al. 2012; 2014; Dias et al. 2011; Peon et al. 2010). These investigations have generated increasing numbers of DHQase structures with high resolution and have significantly advanced the understanding of DHQase catalytic mechanisms (Chaudhuri et al. 1986; Deka et al. 1994; Euverink et al. 1992; Hawkins et al. 1993; Lee et al. 2003; Moore et al. 1993; Roszak et al. 1993).
Type I DHQases catalyze the dehydrogenation of DHQ through a cis-elimination (Chaudhuri et al. 1991; Gourley et al. 1999; Leech et al. 1995), while type II DHQases take a trans-elimination mechanism (Blomberg et al. 2009; Bottomley et al. 1996).

Recent study has revealed that overexpression of DHQase enhanced transformation of quinic acid into shikimic acid in *Glucanobacter oxydans* (Nishikura-Imamura et al. 2014). The kinetic properties of DHQases such as $K_{m}$, $V_{max}$, and $k_{cat}$ are important particularly to design new biocatalysts and to predict the validity and efficiency of newly constructed metabolic networks. So far, only a small number of DHQases from prokaryotes such as *Escherichia coli*, *Mycobacterium tuberculosis*, and *Streptomyces coelicolor* were characterized for their catalytic properties (Harris et al. 1996; Kleanthous et al. 1992; Moore et al. 1993; Richards et al. 2006; White et al. 1990), and the catalytic and kinetic properties of the majority of microbial DHQases remain still unknown.

In this study, we aimed to investigate the kinetic diversities of DHQases and to expand the toolbox of catalytic parts for synthetic biology. By data-mining of 3180 prokaryotic genomes from NCBI genome database, 459 putative DHQases were targeted. Thirty-eight DHQases were further selected and standardized according to “Biobricks” requirements (Knight 2003; Shetty et al. 2011; Sleight et al. 2010). DHQase kinetic constants were determined with a newly established high-throughput method. Our results showed that DHQases are highly diverse in catalytic kinetics.

**Materials and methods**

**Genome data-mining for DHQase genes**

The amino acid sequences of the type II DHQase (NP_599670) from *Corynebacterium glutamicum* ATCC13032 and of the type I DHQase (NP_416208) from *E. coli* K-12 were used as seed sequences to retrieve putative DHQase sequences from NCBI genome database with a filter condition of threshold E value $\leq 10^{-10}$. The retrieved sequences were then screened and redundant copies were removed. To increase the credibility of functional DHQase prediction, the retrieved sequences were further filtered by removal of putative DHQase sequences from which host organisms have incomplete shikimate pathway in their genomes.

**Design and chemical synthesis of DHQase genes**

The selected DHQase amino acid sequences were reverse-translated into DNA sequences, and were recoded with referring codon usage bias of *E. coli* and *C. glutamicum*. The obtained DNA sequences were optimized for expression in *E. coli* and *C. glutamicum* by check for RNA secondary structure with software UNAFold (Markham and Zuker 2008). Any predicted secondary structures were eliminated by codon replacements. The new DHQase genes were further customized, by linking to Biobrick adapters (Knight 2003) at both ends. The customized DNA sequences of the DHQases are accessible at [http://www.genoportal.org/bbdb/](http://www.genoportal.org/bbdb/) under the accession numbers of SBB_00477 ~ SBB_004481, and were chemically synthesized (Sangon Biotech, China), and were cloned in *E. coli*. The DNA sequences of the chemically synthesized DHQase genes were confirmed by DNA sequencing.

**Bacterial strain, plasmids, and growth condition**

For cloning and expression of DHQase genes, *E. coli* BL21 (DE3) (TransGen Biotech, China) and plasmid pET28a* (Novagen, Germany) were used. Expression of DHQase genes was induced with 0.1 mmol/L of IPTG when culture reached OD$_{600nm}$ of 0.6-0.8. After addition of IPTG, culture was further incubated at 16°C, overnight. Routine cultivation of *E. coli* proceeded in Luria-Bertani (LB) medium at 37°C and at 200 rpm rotatory shaking. To maintain the stability of pET28a* and its derivatives, kanamycin at final concentration of 100 μg/ml were added into LB medium.

**Preparation of cellular lysates and purification of DHQase proteins**

*E. coli* cells were harvested by centrifugation at 10,000 rpm, and suspended in 50 mmol/L Tris–HCl buffer, pH 8.0. After addition of 0.01% (w/v) protease inhibitor cocktail (Amresco, the United States), cell suspension was treated with ultrasonication (work for 3 sec, stop for 5 sec, 100 repeats). The cellular debris was removed by centrifugation at 10,000 rpm for 10 min, and the supernatant was filtered with 0.22 μm filters (Millipore, the United States). The filtered supernatant was collected and DHQase proteins were purified using HisPur® Ni-NTA Spin Columns Kit (Thermo Scientific, the United States). The purified DHQase proteins were stored at −80°C in 50 mmol/L Tris–HCl (pH 8.0) buffer containing 25% glycerol. All procedures were operated at 4°C unless indicated.

Protein concentrations were determined with Bio-Rad Protein Assay (BIO-RAD, the UK). Tricine sodium dodecyl sulfate polyacrylamide gel electrophoresis was used to evaluate the DHQase expression and the purity during protein purification. Preparation of gels (4% sample gel and 10% separating gel) and operation of electrophoresis were conducted according to Schägger (2006). Gels were visualized with Coomassie brilliant blue G-250 staining, and were scanned with PC scanner (T68, Founder, China) for image analysis.
DHQase activity assays
A high-throughput method of DHQase activity assay was established in this study. The method has the same principal for measurement as White et al. (1990), but with new systems. Synergy H4 Hybrid Multi-Mode Microplate Reader (BioTek, the UK) and 96 Well UV-Plate (Corning Costar, the United States) were used to monitor the changes of OD\textsubscript{234nm} of multi-samples. DHQase catalysis was optimized in volume of 100 μL and with various DHQ concentrations (0.08 to 1.0 mmol/L). Kinetic constants $K_m$ and $V_{max}$ were calculated according to Lineweaver-Burk plot.

| Sources | DHQase in a genome | Number of genomes |
|---------|--------------------|-------------------|
| Bacteria |                  |                   |
| Type I only |              | 60                |
| Type II only |             | 306               |
| Both Type I and II | | 25                |
| Archaea |                  |                   |
| Type I |                  | 43                |

Notes: Redundant genomes representing the same species were removed.

Table 1 Distribution of type I and type II DHQases in prokaryotic genomes

Results
DHQases are phylogenetically diverse
3180 genomes were explored for putative DHQases, and 459 putative DHQases were targeted, including 128 type I and 331 type II DHQases. Noticeably, all archaeal DHQases were type I. Bacterial genomes carried both type I and type II DHQases, which 79.6% of the bacterial DHQases were type II and 20.4% were type I. Of the 391

Sequence alignments and construction of phylogenetic tree
The amino acid sequences of DHQases were retrieved from NCBI genome database. Amino acid sequence alignment of DHQases were performed with MUSCLE (Edgar 2004), and the graphic display of alignments were made by ESPript3.0 (Gouet et al. 1999). The phylogenetic tree of amino acid sequences from all putative DHQases were firstly constructed by Phylip Package (Abdennadher and Boesch 2007) under Linux system with method of maximum likelihood and bootstrap replications of 1000, and further annotated using iTOL v2.2.2 (Letunic and Bork 2011).
Table 2: Selected DHQase, their origins and theoretical molecular masses of translational products

| Codes for DHQases | Origins | NCBI accession numbers of | Accession IDs at www.genoportal.org | Theoretical molecular mass (kDa) | Sequence identity |
|-------------------|---------|---------------------------|-------------------------------------|----------------------------------|-------------------|
| **Type I**        |         |                           |                                     |                                  |                   |
| 3002              | Escherichia coli | YP_489955.1 | SBB_00449 | 27.5 | 100% |
| 3029              | Staphylococcus aureus | YP_005744190.1 | SBB_00476 | 26.9 | 30.0% |
| 3030              | Metallosphaera cuprina | YP_004409005.1 | SBB_00477 | 23.7 | 23.5% |
| **Type II**       |         |                           |                                     |                                  |                   |
| 3001              | Corynebacterium glutamicum | YP_224725.1 | SBB_00448 | 15.4 | 100.0% |
| 3003              | Alcanivorax borkumensis | YP_693728.1 | SBB_00450 | 17.5 | 50.7% |
| 3004              | Bacillus pseudofirmus | YP_003425282.1 | SBB_00451 | 16.5 | 50.0% |
| 3005              | Citrobacter koseri | YP_001456151.1 | SBB_00452 | 16.5 | 53.4% |
| 3006              | Enterobacter aerogenes | YP_004591144.1 | SBB_00453 | 16.5 | 53.0% |
| 3007              | Glucanobacter oxydans | YP_190876.1 | SBB_00454 | 17.4 | 51.1% |
| 3008              | Mycobacterium tuberculosis | YP_003031384.1 | SBB_00455 | 15.8 | 52.6% |
| 3009              | Psychrobacter sp. PRWf-1 | YP_001279670.1 | SBB_00456 | 18.4 | 52.8% |
| 3010              | Psychromonas ingrahamii | YP_944417.1 | SBB_00457 | 16.5 | 53.0% |
| 3011              | Xylella fastidiosa | NP_297340.2 | SBB_00458 | 16.7 | 52.2% |
| 3012              | Arthrobacter crystallinum | EMY34512.1 | SBB_00459 | 16.3 | 71.9% |
| 3013              | Micromonas pyroidea | CCH17589.1 | SBB_00460 | 15.4 | 47.8% |
| 3014              | Nocardia brasiliensis | YP_006813099.1 | SBB_00461 | 15.6 | 64.0% |
| 3015              | Propionibacterium acnes | YP_056375.1 | SBB_00462 | 15.8 | 43.7% |
| 3016              | Streptomyces acidiscabies | WP_010352491.1 | SBB_00463 | 16.8 | 61.4% |
| 3017              | Thermomonas poracurata | YP_003300530.1 | SBB_00464 | 16.6 | 45.3% |
| 3018              | Atopobium rimae | EEE18006.1 | SBB_00465 | 15.5 | 41.7% |
| 3019              | Acidimicrobium ferrooxidans | YP_003110305.1 | SBB_00466 | 16.4 | 45.8% |
| 3020              | Bacillus subtilis | NP_390327.1 | SBB_00467 | 16.4 | 49.3% |
| 3021              | Geobacillus sp. Y4.1MC1 | YP_00398579.1 | SBB_00468 | 16.0 | 48.6% |
| 3022              | Alicyclobacillus acidocaldarius | EED07485.1 | SBB_00469 | 16.3 | 49.6% |
| 3023              | Butyrivibrio catus | EFF68279.1 | SBB_00470 | 22.8 | 47.1% |
| 3024              | Halanaerobium hydrogeniformans | YP_003995185.1 | SBB_00471 | 16.6 | 51.4% |
| 3025              | Clostridium clarum | YP_005045936.1 | SBB_00472 | 15.5 | 53.1% |
| 3026              | Natrananaerobius thermophilus | YP_001917881.1 | SBB_00473 | 15.8 | 43.7% |
| 3027              | Ruminococcus champanellensis | YP_007829441.1 | SBB_00474 | 16.1 | 49.6% |
| 3028              | Thermodesulfobium narugense | YP_004438087.1 | SBB_00475 | 16.8 | 46.3% |
| 3031              | Comamonas testosteroni | YP_003279351.1 | SBB_00478 | 15.9 | 52.1% |
| 3032              | Pseudomonas putida | NP_745147.1 | SBB_00479 | 16.2 | 59.4% |
| 3033              | Halomonas elongata | YP_004399044.1 | SBB_00480 | 15.5 | 55.8% |
| 3034              | Vibrio vulnificus | NP_935927.1 | SBB_00481 | 18.7 | 55.6% |
| 3035              | Acidithiobacillus caldus | YP_004750222.1 | SBB_00482 | 16.9 | 50.7% |
Table 2 Selected DHQase, their origins and theoretical molecular masses of translational products (Continued)

| Code | Species                  | Accession   | Mass (kDa) | Identity (%) |
|------|--------------------------|-------------|------------|--------------|
| 3036 | Klebsiella pneumoniae    | EMH96211.1  | 16.5       | 51.5%        |
| 3037 | Magnetospirillum         | YP_422069.1 | 14.5       | 50.4%        |
| 3038 | Zymomonas mobilis        | YP_162472.1 | 15.8       | 52.9%        |

The amino acid sequence identities of DHQases to the one from C. glutamicum (type II) or from E. coli (type I) were calculated based on alignment with BLAST searches. The NCBI numbers refer to the wildtype DHQases, and the customized DNA sequences of these DHQases are accessible at http://www.genoportal.org/bibdb.

*The first digit of the code represents DHQase catalyzes the 3rd step of shikimate pathway, and the following digits are the order when that DHQases were selected.

All 38 DHQase genes were cloned with E. coli. Except those DHQases of A. ferrooxidans, B. pseudofirmus, S. aureus and Z. mobilis that occurred as either inclusion bodies or no synthesis in E. coli cells, all other DHQase were synthesized and purified. The purified DHQases actively catalyzed the conversion of DHQ into 3-dehydroshikimic acid.

DHQases have broad ranges of kinetic parameters

Catalytic properties such as $V_{\text{max}}$, $K_m$, $k_{\text{cat}}$, and $k_{\text{cat}}/K_m$ of the selected DHQases were determined and are listed in Table 3. Results showed that their kinetic parameters are distributed in broad ranges, which suggested a kinetic diversity of DHQases regarding catalytic capacity and efficiency, and substrate affinity. The $V_{\text{max}}$ values of DHQases at the given enzyme concentrations in this study were determined to be 0.57-66.7 μmol/L/s. The $K_m$ values were determined to be 37.2-2226.5 μmol/L. These results indicated that natural DHQases had evolved broad catalytic properties, probably for reasons to serve their hosts at different growing conditions. The DHQase (Code 3012) from A. crystallopoietes showed the highest catalytic capacity ($V_{\text{max}} = 66.7 \mu\text{mol/L/s}$) and moderate affinity to 3-dehydroquinate ($K_m = 448.5 \mu\text{mol/L}$). The DHQase (Code 3031) from C. testosteroni showed highest affinity to 3-dehydroquinic acid ($K_m = 37.2 \mu\text{mol/L}$) among the tested DHQases. The kinetic parameter $k_{\text{cat}}$ signifying the catalytic efficiency of an enzyme, and it was found that the DHQase (Code 3016) from S. acidiscabies was the most efficient one among the tested DHQases and its $k_{\text{cat}}$ value was determined to be 211.83 s⁻¹. When all the tested DHQases were evaluated by $k_{\text{cat}}/K_m$ values, the DHQase (Code 3016) had the highest $k_{\text{cat}}/K_m$ value (1.50 L/μmol/s) that represented the best catalytic specificity and efficiency among all the tested DHQases. In addition, the specific activities of DHQases at 3-dehydroquinic acid concentration of 0.5 mM were determined and DHQase (Code 3016) from S. acidiscabies showed the highest activity (501.87 units/mg). Figure 2 depicts a whole image of kinetic parameter distribution of DHQases. Apparently, the kinetic parameters of all tested DHQases varied significantly, reflecting a long-span distribution of their catalytic properties and kinetic diversity.

Cloning and expression of customized DHQase genes in E. coli cells

From all retrieved 459 DHQases, 38 were further selected (Table 2). The selection was carried out according to the following criterions: 1) Both type I and type II DHQases were included, and the majority was bacterial type II DHQases; 2) Host genomes harbored a complete shikimate pathway, or taxonomically representative (Archaee, Actinobacteria, Firmicutes, and Proteobacteria), or from unique habitats. From the 38 DHQases, 35 were of type II, and 3 were of type I. The theoretical molecular masses of type II DHQases were ranged from 15 to 18 kDa, and their amino acid sequence identities to DHQase from C. glutamicum were 41.7-71.9%. The selected DHQases were recorded by referring the codon usages of E. coli and of C. glutamicum (Nakamura 2007). The new DNA sequences were edited by curing restriction endonucleases (NdeI, XhoI, EcoRI, NotI, XbaI, SpeI and PstI) sites and any predicted secondary mRNA structures. Further, the sequences were customized for “Biobricks” (Knight 2003) by attaching the prefix 5′-GCAGAATTTCGCGCCGCCTTCTAGA-3′ and the suffix 5′-ACTAGTAGCGCCGCCTGCAG-3′.

genomes explored, 306 genomes (78.3%) harbored only type II DHQases, 60 genomes (15.3%) harbored only type I, and 25 genomes (6.4%) harbored both type I and type II (Table 1).

To display the phylogenetic relationship of all 459 DHQase sequences and taxonomic distribution, their amino acid sequences were used to construct a phylogenetic tree (Figure 1). As seen from Figure 1, seven clades (from cluster A to G) of DHQases were recognized. The majority of type I DHQases fell into the clade A, which contained two sub-clades $A_1$ and $A_2$. Sub-clade $A_1$ covered bacterial type I DHQases from Proteobacteria, Actinobacteria, and Firmicutes. Sub-clade $A_2$ was mainly archaeal DHQases from Thaumarchaeota, Crenarchaeota, and Eurarchaeota. Clades B through G were type II DHQases of different bacterial phyla. DHQases of the clades C and F were mainly from Actinobacteria and Firmicutes, respectively. DHQases of clades D and G were mainly from Proteobacteria. DHQases of clades B and E were from Proteobacteria, Actinobacteria and other bacterial phyla.
Kinetic diversity of enzymes is the results of natural revolution in life, similar to their phylogenetic diversity (Zhi et al. 2014). But the kinetic diversity of enzymes had not yet been explored before. This study took DHQase as an example to explore the kinetic diversity of enzymes involved in shikimate pathway. The results from this study demonstrated that DHQases from various hosts had very broad ranges of affinity ($K_m$) to substrate, catalytic efficiency and catalytic capacity ($k_{cat}$).

Table 3 The kinetic parameters of 3-dehydroquinate dehydratases

| Codes for DHQases* | $V_{max}$ (μmol/L/s) | $K_m$ (μmol/L) | $k_{cat}$ (s$^{-1}$) | $k_{cat}/K_m$ (L/μmol/s) | Specific Activity** (U/mg) |
|--------------------|----------------------|----------------|---------------------|--------------------------|--------------------------|
| 3001               | 2.56                 | 281.43         | 52.26               | 0.19                     | 119.14                   |
| 3002               | 4.57                 | 187.68         | 29.53               | 0.16                     | 57.34                    |
| 3003               | 1.34                 | 210.71         | 13.53               | 0.06                     | 34.72                    |
| 3004               | —                    | —              | —                   | —                        | —                        |
| 3005               | 15.50                | 870.37         | 208.96              | 0.24                     | 93.91                    |
| 3006               | 6.39                 | 478.67         | 102.15              | 0.21                     | 187.69                   |
| 3007               | 2.43                 | 777.75         | 77.06               | 0.10                     | 84.95                    |
| 3008               | 2.46                 | 624.52         | 23.96               | 0.04                     | 46.37                    |
| 3009               | 4.24                 | 203.24         | 31.67               | 0.16                     | 75.06                    |
| 3010               | 0.80                 | 202.55         | 10.59               | 0.05                     | 9.39                     |
| 3011               | 0.57                 | 158.79         | 7.15                | 0.05                     | 15.06                    |
| 3012               | 66.67                | 448.50         | 185.43              | 0.41                     | 369.99                   |
| 3013               | 7.04                 | 159.89         | 4.63                | 0.03                     | 86.50                    |
| 3014               | 3.60                 | 151.51         | 3.83                | 0.03                     | 91.93                    |
| 3015               | 4.18                 | 46.14          | 10.00               | 0.22                     | 67.71                    |
| 3016               | 12.42                | 141.53         | 211.83              | 1.50                     | 501.87                   |
| 3017               | 8.25                 | 1218.56        | 11.92               | 0.01                     | 30.19                    |
| 3018               | 0.68                 | 400.82         | 9.06                | 0.02                     | 15.64                    |
| 3019               | —                    | —              | —                   | —                        | —                        |
| 3020               | 0.96                 | 107.07         | 10.46               | 0.10                     | 62.27                    |
| 3021               | 2.16                 | 133.97         | 5.33                | 0.04                     | 15.90                    |
| 3022               | 8.26                 | 601.64         | 119.98              | 0.20                     | 195.81                   |
| 3023               | 2.86                 | 67.88          | 42.20               | 0.62                     | 68.99                    |
| 3024               | 6.23                 | 526.07         | 75.11               | 0.14                     | 131.08                   |
| 3025               | 5.89                 | 368.46         | 59.61               | 0.16                     | 117.89                   |
| 3026               | 2.95                 | 304.54         | 3.80                | 0.01                     | 5.16                     |
| 3027               | 6.00                 | 255.54         | 50.20               | 0.20                     | 116.07                   |
| 3028               | 1.16                 | 180.72         | 6.88                | 0.04                     | 17.66                    |
| 3029               | —                    | —              | —                   | —                        | —                        |
| 3030               | 1.14                 | 92.76          | 3.86                | 0.04                     | 8.58                     |
| 3031               | 1.45                 | 37.16          | 5.95                | 0.16                     | 19.82                    |
| 3032               | 12.35                | 617.28         | 78.87               | 0.13                     | 105.18                   |
| 3033               | 2.64                 | 150.25         | 57.84               | 0.38                     | 155.93                   |
| 3034               | 3.78                 | 345.90         | 37.97               | 0.11                     | 71.87                    |
| 3035               | 11.14                | 2226.50        | 192.27              | 0.09                     | 136.57                   |
| 3036               | 9.64                 | 131.63         | 102.70              | 0.78                     | 192.10                   |
| 3037               | 3.87                 | 119.97         | 73.18               | 0.61                     | 365.73                   |
| 3038               | —                    | —              | —                   | —                        | —                        |

*The first digit of the code represents DHQase catalyzes the 3rd step of shikimate pathway, and the following digits are the order when that DHQase was selected.

**Specific activities were determined at substrate concentration of 0.5 mM. "-" indicated no activity was detected.

Discussion
Kinetic diversity of enzymes is the results of natural revolution in life, similar to their phylogenetic diversity (Zhi et al. 2014). But the kinetic diversity of enzymes had not yet been explored before. This study took DHQase as an example to explore the kinetic diversity of enzymes involved in shikimate pathway. The results from this study demonstrated that DHQases from various hosts had very broad ranges of affinity ($K_m$) to substrate, catalytic efficiency and catalytic capacity ($k_{cat}$).
and $V_{\text{max}}$). It was considered that other enzymes of the shikimate pathway would also have broad ranges of kinetic constants. Such broad ranges of kinetic constants are reflections of natural evolution and adaptation of enzymes, and founded the cornerstones of diverse metabolic fluxes in different lives. Therefore, it would be theoretically feasible to engineer metabolic processes quantitatively by mining enzymatic kinetic database. Such strategy is basically different from the current used strategy, i.e., manipulation at levels of genetic transcription and translation (Rytter et al. 2014; Salis et al. 2009; Sohoni et al. 2014).

Recently, synthetic biology has emerged as a new discipline to manipulate biological systems for application and to understand the law of life (Andrianantoandro et al. 2006; Cheng and Lu 2012). The basic units for synthetic biology are standardized biological modules and parts, and standardized enzymes serve as catalytic parts for assembling novel and artificially designed biological systems (Cooling et al. 2010). Such catalytic part library is represented by the chemically synthesized genes of methyl halide transferases (Bayer et al. 2009). In this study, 38 selected DHQase genes were chemically synthesized and customized as Biobricks for future applications. All DHQases have Biobrick adapters, so they are compatible to other Biobrick parts/modules. Our study is part of an on-going project of design and construction of artificial modules for shikimate pathway using synthetic biological tools, aiming to create modules for shikimate pathway that would stimulate industrial applications for bioproduction of aromatic-related primary and secondary compounds. The pipeline for DHQase sequence design, chemical synthesis and purification established from this study has been generalized for other enzymes of the shikimate pathway, and construction of a catalytic part library of shikimate pathway is in progress.

Previous reports described that type I DHQases are represented by DHQases from plants and fungi (Hawkins et al. 1993; Weaver and Herrmann 1997), we have found that all 43 archaeal DHQases in this study were belonging to type I, although this study was focused on type II DHQases. Type II DHQases had been classified into two groups in terms of their kinetic constants $k_{\text{cat}}$ (Pan et al. 2012). The first group included DHQase from S. coelicolor and had relative high $k_{\text{cat}}$ values larger than $100 \text{ s}^{-1}$, whereas the second group...
included DHQase from *M. tuberculosis* with a $k_{cat}$ lower than 10 s$^{-1}$. As demonstrated in this study, the $k_{cat}$ values of DHQases distributed continuously from 4 to over 200 s$^{-1}$, which rendered it unrealistic to deivide them into two groups based on $k_{cat}$ values.

Alignments of type II DHQases revealed a number of conserved amino acid residues that are potentially important for catalytic efficiency and capacity. Specifically, the Tyr24 residue facilitated the proton abstraction from substrate, and then the His101 residues promoted the dehydrogenation by donating proton to 1-hydroxyl on C1 of substrate as a general acid (Blomberg et al. 2009; Pan et al. 2012). The residue His101 was found in all of the type II DHQases. But Tyr24 was replaced by Phe residue in DHQase from *B. subtilis* (Code 3020) (Data not shown). It was found that this DHQase (Code 3020) were functional and catalyzed the conversion of DHQ to 2-dehydroshikimate. The Phe residue was found in eighteen *B. subtilis* genomes as well as other bacilli genomes such as *B. mojavensis, B. tequilensis, B. vallismortis, B. atrophaeus* and *B. tequilensis*. These 18 DHQases were functional and catalyzed the conversion of DHQ to 2-dehydroshikimate. Thus, the catalytic mechanism of the Phe-DHQase has not been explored. Since Phe residue is rather stable and hard to be deprotonated, the catalytic mechanism of the Phe-DHQases might be different from that of the previous characterized DHQases.

**Competing interests**

The authors declare that they have no competing interests.

**Authors’ contributions**

CL carried out the studies of gene cloning and expression, enzyme purification and activity determination. YL carried out the sequence analysis, gene recoding and participated sequence analysis. Both CYJ and CL helped to draft the manuscript. SJL conceived of the study and finalized the manuscript. All authors read and approved the final manuscript.

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