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A genome-based approach to create a minimally mutated *Corynebacterium glutamicum* strain for efficient L-lysine production

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Abstract

Based on the progress in genomics, we have developed a novel approach that employs genomic information to generate an efficient amino acid producer. A comparative genomic analysis of an industrial L-lysine producer with its natural ancestor identified a variety of mutations in genes associated with L-lysine biosynthesis. Among these mutations, we identified two mutations in the relevant terminal pathways as key mutations for L-lysine production, and three mutations in central metabolism that resulted in increased titers. These five mutations when assembled in the wild-type genome led to a significant increase in both the rate of production and final L-lysine titer. Further investigations incorporated with transcriptome analysis suggested that other as yet unidentified mutations are necessary to support the L-lysine titers observed by the original production strain. Here we describe the essence of our approach for strain reconstruction, and also discuss mechanisms of L-lysine hyperproduction unraveled by combining genomics with classical strain improvement.

Key words amino acid fermentation · metabolic engineering · classical mutagenesis · genomics · L-lysine · *Corynebacterium glutamicum*

Introduction

L-Amino acids, the basic building blocks of proteins, are used in a variety of fields such as food, medicine, and chemicals. The annual world production of L-amino acids has annually increased, and is currently estimated at 2 million metric tons. Most L-amino acids are manufactured by fermentation using *Corynebacterium glutamicum* [7, 11]. This amino acid fermentation was developed primarily in Japan and today is a global industry. However, this market is highly competitive and process economics are of primary importance. For cost-effective production, innovation in fermentation technologies has become an urgent need in industry. The present review describes a new technology for strain development that employs genome information and its application to the improvement of L-lysine fermentation as a model.

Production strains used in industrial fermentation processes have traditionally been constructed by repeating random mutation and selection [1, 7, 11]. In this approach, chemical mutagens (e. g., nitrosoguanidine) or ultraviolet are used to induce mutants

that exhibit improved production. The drawback of this method is its inability to avoid introducing secondary mutations into the genome. Because of this, detrimental or unnecessary mutations are presumed to accumulate in one background over the course of many cycles of mutagenesis. Consequently, production strains generally have weak constitutions, as illustrated by their poor growth and sugar consumption compared to corresponding wild-type strains. In addition, the causative mutations are undefined. This leaves their mechanisms ambiguous and hinders their rational exploitation using metabolic engineering. If all the purported unnecessary mutations could be eliminated from the genome of a classically improved strain, one would predict that resulting performance would be substantially improved. Such an improvement would likely have a significant positive impact on conventional fermentation processes.

A methodology that aims at such engineering is the "genome-based strain reconstruction" shown in Fig. 1. In this approach, only beneficial mutations identified by analysis of a producer genome are assembled in a single wild-type background. By doing so, it becomes possible to create a defined mutant that carries a minimal set of essential mutations for high-level production and, at the same time, to understand the production mechanisms. It should be noted that this methodology can be clearly distinguished from the conventional method, because the former introduces specific mutations with intended genotypes while the latter selects improved strains by phenotypes.

The whole-genome sequence of the wild-type strain *C. glutamicum* ATCC 13032 is available under the accession numbers, BA000036 (Kyowa Hakko and Kitasato University) and BX927147 (Degussa AG and Bielefeld University). The progress in genomics, genetics, biochemistry, physiology, and applications of *C. glutamicum* has been reviewed [2, 5, 7, 8, 10, 11].

Reconstructing an L-lysine producer according to genome information

Reconstruction of an L-lysine producer is carried out in the following way [8, 9, 14-16]. First, all predicted genes relevant to L-lysine biosynthesis are identified from the genome and organized into a metabolic map, as shown in Fig. 2. Next, the sequences of these same genes are determined for a classically derived L-lysine producer and are compared with the wild-type sequences to identify mutational differences. The mutations

are then sequentially introduced by allelic replacement into the wild-type genome. The replacement starts with mutations in the relevant terminal pathways and moves to those in central metabolic pathways, with each construct evaluated for its contribution to production. When the mutation is relevant to production, it is saved in the genome, and the resulting mutant is used as the parent to introduce and evaluate a second mutation. This iterative cycle makes it possible to generate a minimally mutated strain consisting of only relevant mutations. An example is illustrated below.

Mutating the terminal pathways

Sequence comparison of alleles in the relevant terminal pathways from L-aspartate with their wild-type counterparts revealed that the *hom*, *lysC*, *dapE*, *dapF*, *thrB*, and *thrC* genes of a classically derived L-lysine producer *C. glutamicum* B-6 [6] each had a point mutation in the coding region (Fig. 2). These six specific mutations were separately introduced by allelic replacement into the wild-type strain that produces no L-lysine, followed by evaluation of their contribution to production. The *hom* mutation (V59A, designated *hom59*) and the *lysC* mutation (T311I, designated *lysC311*) were found to be relevant to L-lysine production. Comparative phenotypic analyses also revealed that the *hom* and *lysC* mutations conferred on the host the phenotypes of a partial requirement for L-homoserine and of resistance to an L-lysine structural analog, *S*-(2-aminoethyl)-L-cysteine (AEC), respectively. The two mutations were then combined in the wild-type genome, resulting in an additive effect on production. In a fed-batch fermentation using a glucose-containing medium, the *hom-lysC* double mutant AHD-2 produced about 70 g/l of L-lysine, while the *hom* single mutant HD-1 and the *lysC* single mutant AK-1 accumulated about 10 and about 50 g/l of L-lysine, respectively (Fig. 3). The wild-type strain produced no detectable L-lysine.

Mutating central carbon metabolism

After bottlenecks in the terminal pathways are removed, the next goal is to increase the supply of L-aspartate (Fig. 2). Thus, mutations identified in central carbon metabolism were separately introduced into the *hom-lysC* double mutant, AHD-2, followed by evaluation of their effects on L-lysine production. Three specific mutations, *pyc458*

(P458S) in the anaplerotic pathway, *gnd361* (S361F) in the pentose phosphate pathway, and *mqo224* (W224opal) in the TCA cycle, were defined as positive mutations for improved production (Fig. 2). These three mutations were then introduced one by one into the AHD-2 genome, which resulted in step-wise increases in final L-lysine titers (Fig. 3). Any of these engineered strains grew and consumed glucose almost as fast as the wild-type strain, thus leading to completion of fermentations within 30 h, while the classical producer B-6 required more than 50 h to complete fermentation (data not shown).

Rationalizing the mechanism of L-lysine production

The occurrence of L-lysine production by introduction of the *lysC311* mutation, an AEC-resistant mutation, is most likely due to partial deregulation of aspartokinase by L-lysine and L-threonine [16]. On the other hand, the *hom59* mutation, an L-homoserine-leaky mutation, allows L-lysine accumulation presumably by reducing the intracellular pool of L-threonine to a level at which synergistic inhibition of aspartokinase does not tightly occur [16]. The reason why the two mutations exerted an additive effect on production upon coexistence in the wild-type genome can be explained by higher deregulation of aspartokinase [16].

Regarding the *pyc458* mutation in pyruvate carboxylase, it seems reasonable that an improved reaction from pyruvate to oxaloacetate allows for increased availability of pyruvate for L-lysine biosynthesis, although no enzymatic evidence to support the speculation has been obtained [16].

The *gnd361* mutation in 6-phosphogluconate dehydrogenase was found by enzymatic analysis to alleviate the allosteric regulation of the enzyme by intracellular metabolites, such as fructose 1,6-bisphosphate, D-glyceraldehyde 3-phosphate, phosphoribosyl pyrophosphate, ATP, and NADPH [15]. Due to this effect, it was shown by metabolic flux analysis that the producer bearing the *gnd361* mutation exhibited about 8% increased carbon flux through the pentose phosphate pathway during L-lysine production. Considering that NADPH required for L-lysine biosynthesis is chiefly supplied by the pentose phosphate pathway in this bacterium, the mechanism for increased L-lysine production can be rationalized by increased efficiency of NADPH supply.

On the other hand, the *mqo224* mutation in malate:quinone oxidoreductase (MQO) is a nonsense mutation, suggesting that the disruption of the enzyme reaction might be useful for L-lysine production. In fact, a similar effect was obtained by the deletion of the gene encoding MQO, which catalyzes the same reaction as malate dehydrogenase (MDH), the oxidation of malate to oxaloacetate. However, in contrast to NAD-dependent MDH, MQO uses quinines (not NAD) as the electron acceptors. Molenaar et al. [12, 13] investigated the physiological function of the existence of both MQO and NAD-dependent MDH in *C. glutamicum*, and they reported that MQO catalyzes the cyclic conversion of malate and oxaloacetate in cooperation with MDH, leading to a net oxidation of NADH. On the basis of these observations, they suggested that MQO may allow cells to attain a high TCA cycle flux independently of the NADH/NAD ratio. Considering those findings, it is reasonable to postulate that MQO disruption may affect the NADH/NAD ratio to restrict the TCA cycle flux. Since the producer with a deletion in *mqo* exhibited behavior suggesting the coordinate down-regulation of the TCA cycle genes in our DNA array experiment, it seems likely that MQO disruption down-regulates the flux of the TCA cycle to maintain the redox balance and results in redirection of oxaloacetate into L-lysine biosynthesis.

Verifying the performance of the minimally mutated strain

The new strain shown here inherits the robustness of the wild-type strain, and thus has several advantages over classically derived producers. One is its high rate of growth and sugar consumption. Since the new strain can grow and consume glucose almost as fast as the wild-type strain, the fermentation period can be shortened to nearly half of that traditionally required [16]. This is expected to significantly benefit overall productivity as well as production capacity without the need of new fermentors. A second is improved stress-tolerance, which has allowed fermentation at higher temperatures than is traditionally practiced. The new strain showed efficient L-lysine production at 40ºC, whereas classical strains could not function above 35ºC [14]. This feature not only allows reduction of cooling costs, but also enables cost-effective manufacture in tropical regions that have easy access to low-cost carbon sources such as molasses and tapioca. Figure 4 shows rapid growth of new strain AGM-5 at the usual 30ºC temperature as well as at the suboptimal 38ºC temperature, as compared with the classical strain B-6.

Towards further improvement

While verifying the usefulness of this methodology, we found that industrial levels of L-lysine production (more than 100 g/liter) were not attained by our reconstructed strain, suggesting the presence of other unknown mechanisms for production. To address them, we investigated the transcriptome of industrial L-lysine producer B-6 [4]. When total RNAs from mid-exponential phase cultures of 5-liter jar fermentors were used to study differential transcription profiles between strain B-6 and its parental wild-type, it was revealed that many amino acid-biosynthetic genes including the *lysC-asd* operon, the key genes for L-lysine biosynthesis, were up-regulated in the producer (Fig. 5). We next examined whether those global expression changes occurred in new strain AGM-5 generated by our approach. However, we could not observe significant global expression changes for the amino acid-biosynthetic pathways in strain AGM-5 [4]. These results suggest that a certain global regulatory mechanism is involved in the industrial levels of L-lysine production. Thus, we now assume that the global induction of the amino acid-biosynthetic genes might contribute to the increased L-lysine production through increased expression of *lysC-asd*.

Conclusions and perspectives

The genome-based approach presented here is an example of promptly demonstrating the methodology to apply the fruits of genomics to biotechnological processes. Throughout this work, it was illustrated that strain development based on the specific incorporation of biotechnologically useful mutations in a robust wild-type strain can substantially improve producer performance.

In spite of extensive knowledge on the mechanisms of amino acid production, most of the industrial producers are still veiled in mystery. In this work, we used DNA arrays to characterize the transcriptome of such an industrial producer for the first time [4]. The discovery of the global expression changes in industrial producer B-6 gave us an important clue for unraveling the industrial levels of L-lysine production. We are currently pursuing the relevant mutation(s) from among mutations identified in the genome of strain B-6, revealing additional positive mutations in other amino acid biosynthetic pathways and

also global regulatory genes. After this, by defining the genetic background for high-level production, hitherto unknown mechanisms for amino acid hyperproduction may emerge, which will provide new and valuable insights for future strategies of metabolic engineering.

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Fig. 1 Methodology to create a minimally mutated strain. Beneficial mutations (*stars*) relevant to amino acid production are indicated together with unnecessary mutations (*x*)

Fig. 2 L-Lysine-biosynthetic pathways and the relevant genes in *C. glutamicum*. The five positive mutations identified are indicated above the corresponding gene symbols

Fig. 3 Schematic diagram of the creation of the defined L-lysine producers and their abilities of L-lysine production. Cultivations for L-lysine production were carried out in 5-l jar fermentors using a medium consisted of (per liter) 50 g of glucose, 20 g of corn steep liquor, 25 g of NH_4Cl, 1 g of urea, 2.5 g of KH_2PO_4, 0.75 g of $MgSO_4 \cdot 7H_2O$, and some trace elements and vitamins (pH 7.0). After the sugar initially added was consumed, a solution containing 50% (wt/vol) glucose, 4.5% (wt/vol) NH_4Cl, and 0.5 mg/liter D-biotin was continuously fed until the total amount of sugar in the medium reached 25% [16]

Fig. 4 Comparison of growth of the minimally mutated strain AGM-5 and the classically derived industrial producer B-6 at 30°C and 38°C. Both strains were streaked on MM plates, followed by incubation for 2 days at the temperatures indicated. The classical strain B-6 showed no growth at 38°C

Fig. 5 mRNA levels of amino acid-biosynthetic pathway genes in industrial strain B-6 relative to those in wild-type ATCC 13032. *Reddish boxes* represent genes the expression of which was increased, and *green boxes* represent genes the expression of which was decreased in strain B-6. Genes with the P values >0.001 are represented by *gray boxes*. The magnitude of up-regulation or down-regulation is shown by a color bar. Graphic representations were made by Genespring software (Silicon Genetics, Redwood city, CA). Symbols for genes and gene accession numbers are as in the previous report [3]

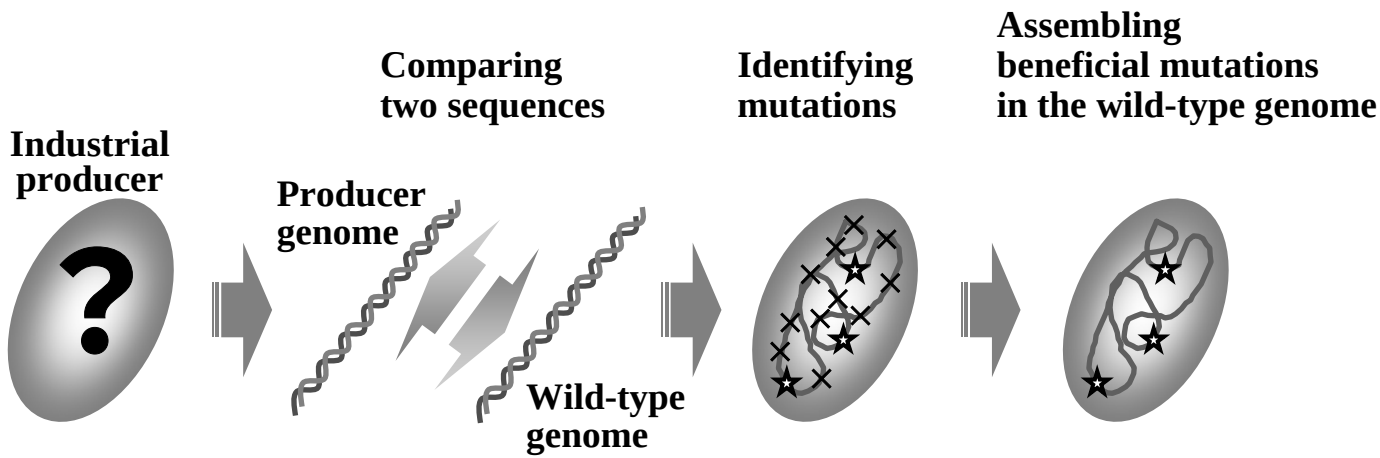
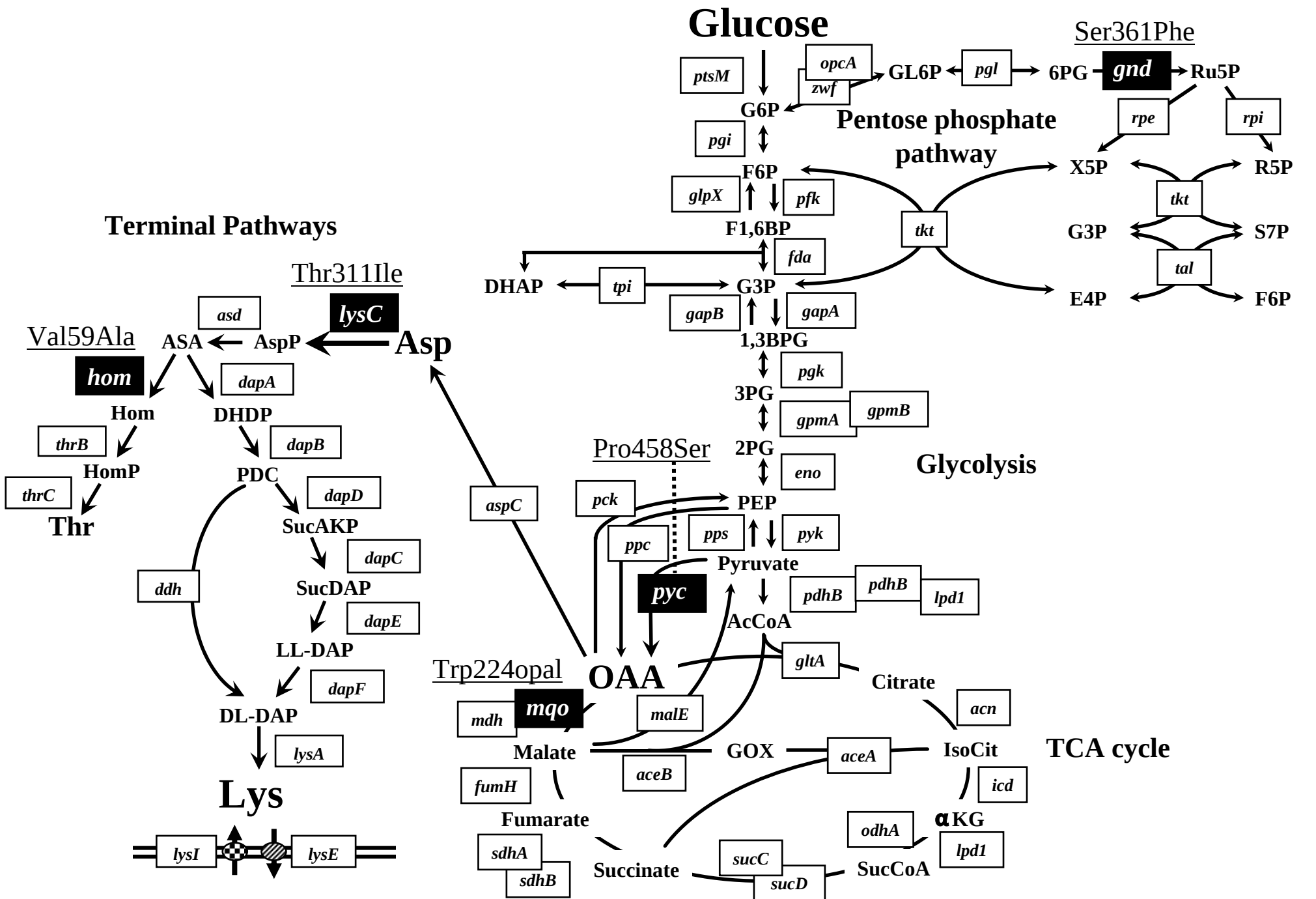

Industrial producer
?
Producer genome
Comparing two sequences
Wild-type genome
Identifying mutations
Assembling beneficial mutations in the wild-type genome

Fig. 1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zwf
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gnd
Ru5P
G6P
rpe
rpi
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pgi
F6P
X5P
R5P
glpX
pfk
tkt
F1,6BP
G3P
S7P
tkt
fda
tal
DHAP
tpi
G3P
E4P
F6P
gapB
gapA
1,3BPG
Terminal Pathways
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asd
lysC
Asp
Val59Ala
ASA
AspP
pgk
hom
dapA
3PG
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DHDP
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gpmB
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Thr
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pdhB
lpd1
ddh
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AcCoA
dapE
gltA
LL-DAP
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OAA
Citrate
dapF
mqo
malE
acn
DL-DAP
mdh
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aceB
icd
fumH
Fumarate
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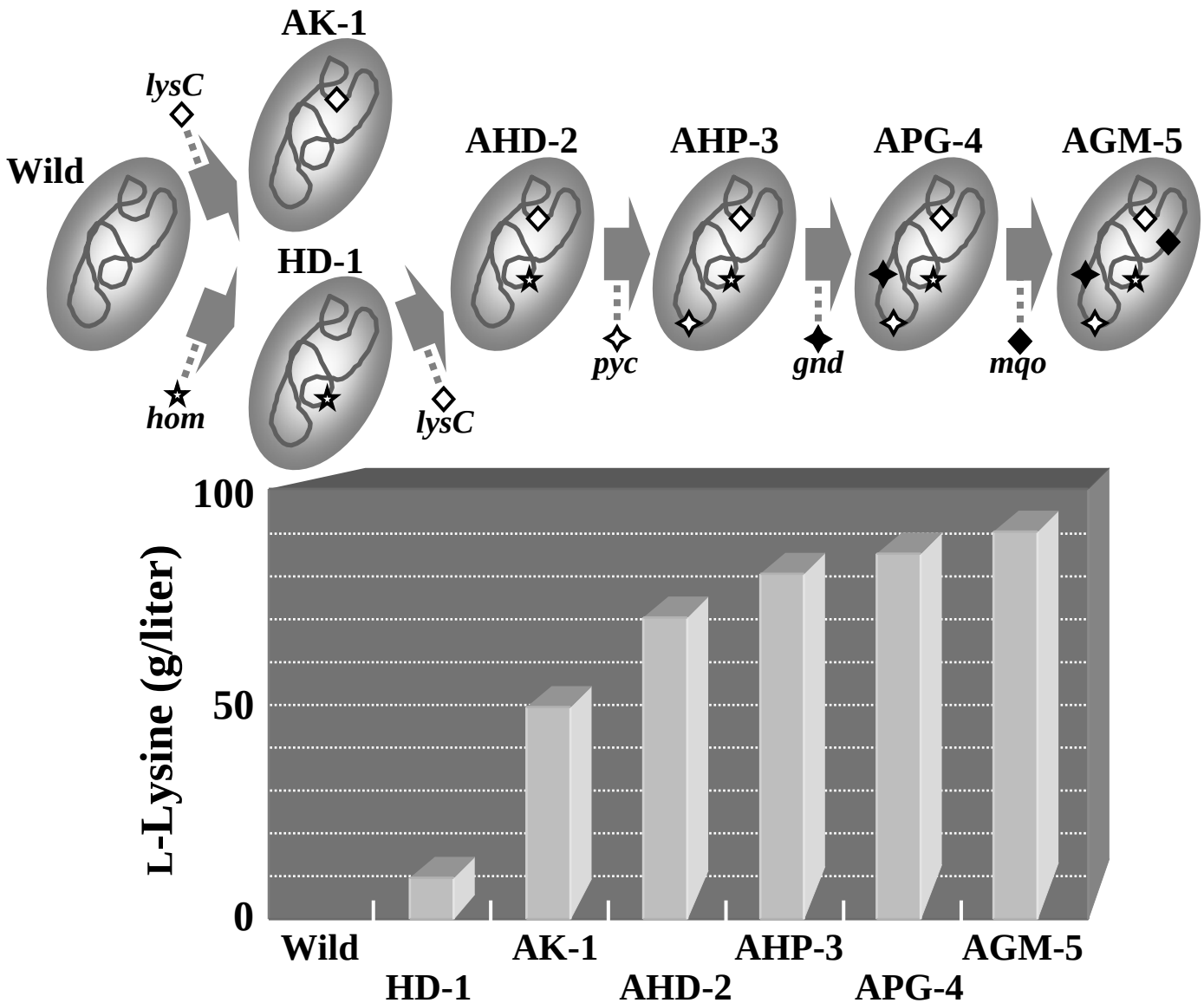
sucD

Fig. 2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lysC
HD-1
hom
lysC
AHD-2
pyc
AHP-3
gnd
APG-4
mqo
AGM-5
L-Lysine (g/liter)
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50
0
Wild
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AK-1
AHD-2
AHP-3
APG-4
AGM-5

Fig. 3

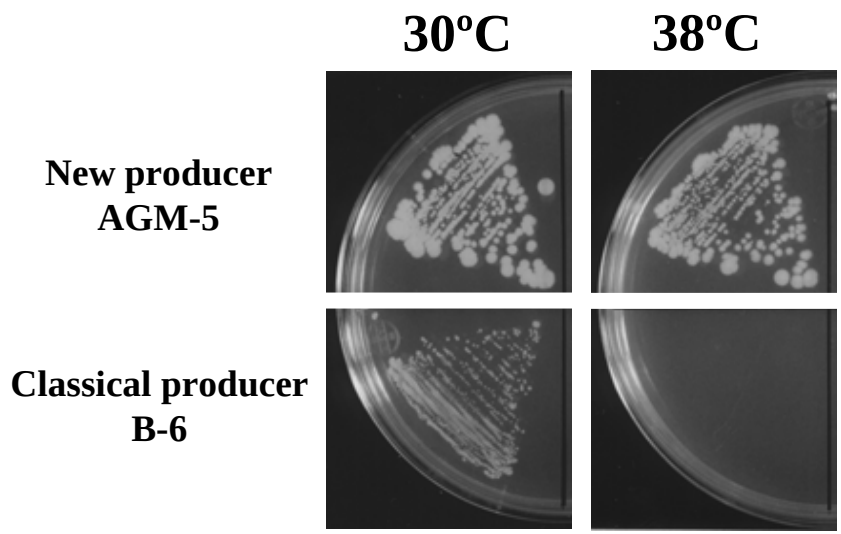
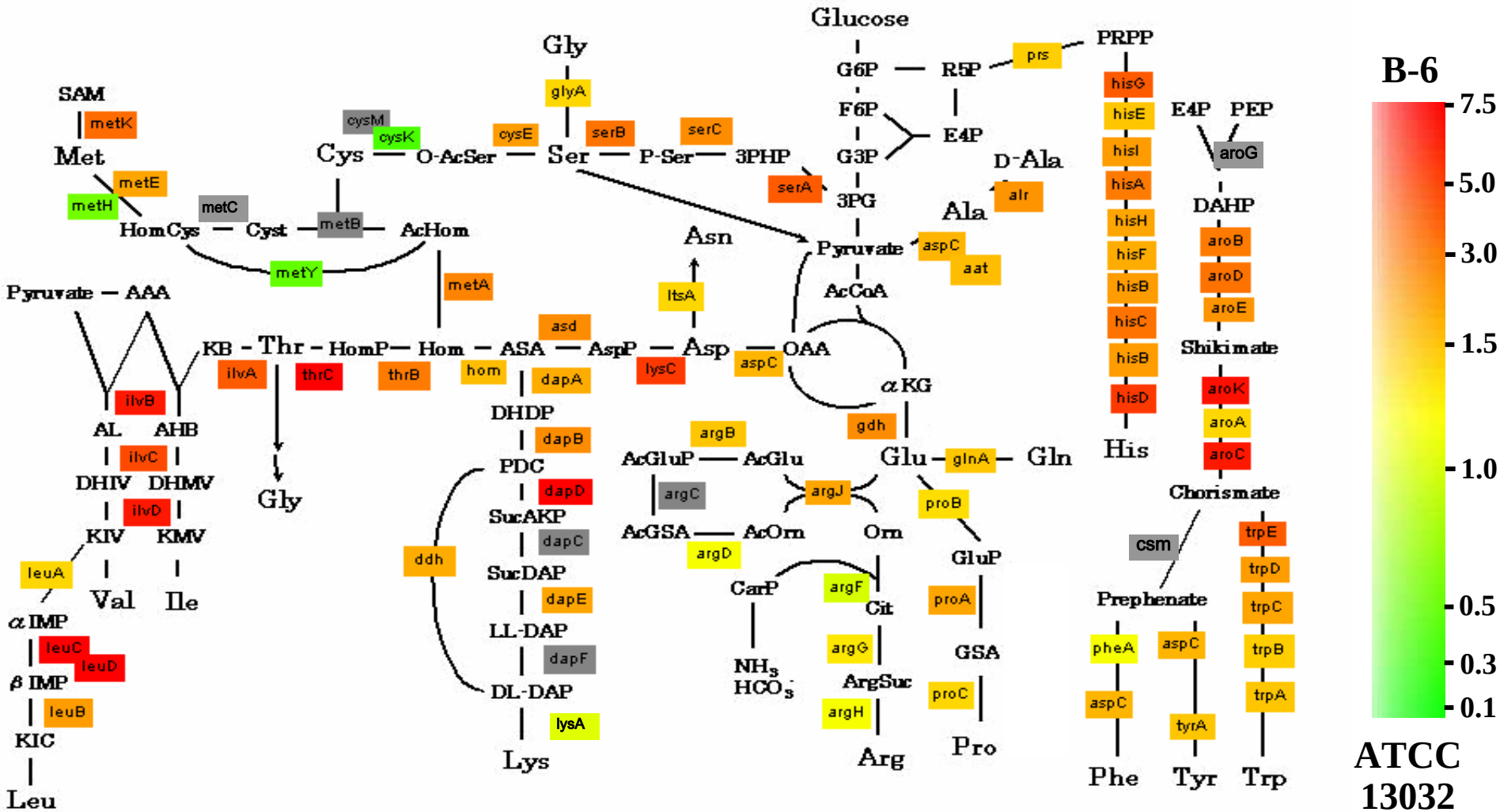


Fig. 4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F6P
E4P
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3PG
Gly
glyA
SAM
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metC
Cys
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cysK
cysE
O-AcSer
Ser
serB
serC
P-Ser
3PHP
serA
D-Ala
alr
Ala
HomCys
Cyst
metB
AcHom
metY
metA
Asn
ltsA
Pyruvate
aspC
aat
AcCoA
Pyruvate — AAA
KB - Thr - HomP - Hom - ASA - AspP - Asp - OAA
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thrB
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αKG
gdh
ilvB
AL
AHB
DHDP
dapB
argB
AcGluP
AcGlu
Glu
glnA
Gln
ilvC
DHIV
DHMV
PDC
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argC
argJ
proB
Gly
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Val
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dapF
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DAHP
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aroD
aroE
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aroK
aroA
aroC
Chorismate
csm
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trpC
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trpA
Prephenate
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aspC
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Phe
Tyr
Trp
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ATCC
13032

Fig. 5