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ORIGINAL PAPER

J. Ohnishi · S. Mitsuhashi · M. Hayashi · S. Ando
H. Yokoi · K. Ochiai · M. Ikeda

A novel methodology employing *Corynebacterium glutamicum* genome information to generate a new L-lysine-producing mutant

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Abstract Classical whole-cell mutagenesis has achieved great success in development of many industrial fermentation strains, but has the serious disadvantage of accumulation of uncharacterized secondary mutations that are detrimental to their performance. In the post-genomic era, a novel methodology which avoids this drawback presents itself. This "genome-based strain reconstruction" involves identifying mutations by comparative genomic analysis, defining mutations beneficial for production, and assembling them in a single wild-type background. Described herein is an initial challenge involving reconstruction of classically derived L-lysine-producing *Corynebacterium glutamicum*. Comparative genomic analysis for the relevant terminal pathways, the efflux step, and the anaplerotic reactions between the wild-type and production strains identified a Val-59→Ala mutation in the homoserine dehydrogenase gene (*hom*), a Thr-311→Ile mutation in the aspartokinase gene (*lysC*), and a Pro-458→Ser mutation in the pyruvate carboxylase gene (*pyc*). Introduction of the *hom* and *lysC* mutations into the wild-type strain by allelic replacement resulted in accumulation of 8 g and 55 g of L-lysine/l, respectively, indicating that both these specific mutations are relevant to production. The two mutations were then reconstituted in the wild-type genome, which led to a synergistic effect on production (75 g/l). Further introduction of the *pyc* mutation resulted in an additional contribution and accumulation of 80 g/l after only 27 h. This high-speed fermentation achieved the highest productivity (3.0 g l^{-1} h^{-1}) so far reported for microbes producing L-lysine in fed-batch fermentation.

J. Ohnishi · S. Mitsuhashi · M. Hayashi · S. Ando · H. Yokoi
K. Ochiai · M. Ikeda (✉)
Tokyo Research Laboratories, Kyowa Hakko Kogyo Co., Ltd.,
Asahi-machi, Machida, Tokyo 194-8533, Japan
e-mail: m.ikeda@kyowa.co.jp
Fax: +81-42-7268330

Introduction

Production strains that are used in industrial fermentation processes of useful metabolites, including amino acids, nucleotides, vitamins, and antibiotics, have generally been constructed by repeated random mutation and selection (Demain 2000). In recent years, many examples of strain improvement using molecular techniques have been reported (Ikeda and Katsumata 1992, 1999; Katsumata and Ikeda 1993; Leuchtenberger 1996). In such cases, however, most of the parent strains to be improved are themselves mutants derived from the mutagenic procedure. Although this classical method has contributed greatly to the progress of the fermentation industry, it has the serious disadvantage of leading to accumulation of secondary mutations that negatively affect the performance of potentially powerful production organisms. Because of this, classically derived production mutants are generally inferior to corresponding wild-type strains in industrially important properties such as growth, sugar consumption, and stress tolerance, which hampers establishment of highly productive industrial processes. In addition, numerous undefined mutations leave the production mechanism ambiguous, which hinders subsequent rational metabolic engineering. Therefore, strain improvement which would eliminate all detrimental or unnecessary mutations from the genome would be a very promising strategy. However, such engineering has so far been effectively impossible using conventional molecular approaches because the uncharacterized mutagen-induced mutations have a genome-wide distribution.

The availability of genomic data from industrial organisms allows PCR-based cloning and sequencing of any desired genes of production strains derived from these organisms. By comparing sequences from wild-type and classically derived production strains, it should be possible to decipher the results obtained from mutation-selection and define the genetic background that is required to achieve high-level production. Subsequently, a methodology of assembling only those mutations bene-

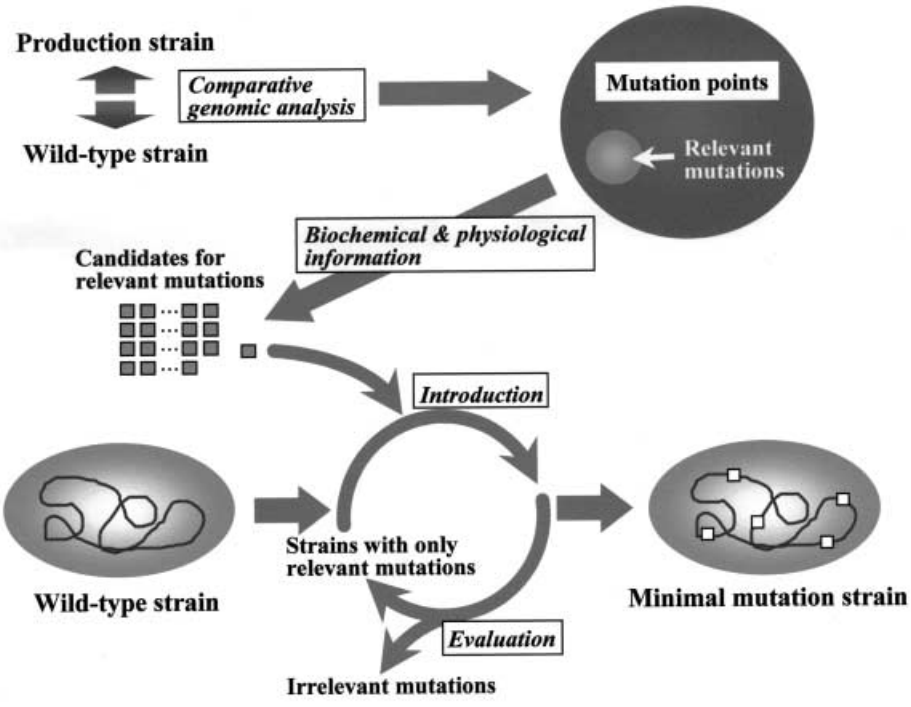

Fig. 1 Methodology of genome-based reconstruction of a classically derived production strain. Candidates for the relevant mutations are introduced one by one from the relevant terminal pathways to central metabolism into the wild-type genome by allelic replacement. Only the relevant mutations (*open squares*) are saved to generate a defined mutant with the minimal mutation set that is necessary and sufficient for high-level production (minimal mutation strain)

ficial for production in a single wild-type background could established a novel approach to exclude the above-mentioned drawbacks of the classical method. This "genome-based strain reconstruction" can be carried out according to the scheme shown in Fig. 1. Working from comparative genomic analysis, along with known biochemical and physiological information, it is possible to specify potentially relevant mutations from among those identified. One of these candidate mutations is introduced into the wild-type genome by allelic replacement, followed by evaluation of its contribution to production. When the mutation is relevant to production, it is saved in the wild-type genome, and the resulting mutant is in turn used as the parent strain to introduce and evaluate a second mutation. The effectiveness of a mutation positioned metabolically upstream may be masked by downstream pathways if other significant rate-limiting steps exist. Hence, it is preferred to first mutate the late steps of a pathway, followed by introduction of mutations that affect earlier steps, specifically from the relevant terminal pathways to central metabolism, so that the limited carbon flow can be released from the nearest point to the end product. This iterative cycle makes it possible to generate a defined production strain carrying a minimal set of essential mutations for high-level production – a "minimal mutation strain" – in parallel with characterizing the genetic background.

Our laboratories just recently achieved complete determination of the whole-genome sequence (Nakagawa et al. 2001) of *Corynebacterium glutamicum* (ATCC 13032), an industrially important organism that is most widely used for the production of various amino acids (Kinoshita and Nakayama 1978). Following the completion of the genome sequence as well as the progress in gene manipulation technology in this organism (Jetten and Sinskey 1995), we have started to reconstruct a classically derived L-lysine producer of *C. glutamicum*. Described herein is the initial challenge undertaken towards genome-based strain reconstruction.

Materials and methods

Bacterial strains and plasmids

The L-lysine-producing strain used for comparative genomic analysis is *C. glutamicum* B-6 (Hirao et al. 1989), which was derived by multiple rounds of mutagenesis from the wild-type strain *C. glutamicum* ATCC 13032. This production strain has many mutations that lead to resistance to an L-lysine structural analog, *S*-(2-aminoethyl)-L-cysteine (AEC), rifampicin, streptomycin, and 6-azauracil. *C. glutamicum* ATCC 13032 was used as a host for reconstitution of beneficial mutations. *Escherichia coli* DH5α was used as a host for cloning of PCR products. Plasmid pESB30, which is nonreplicative in *C. glutamicum*, is a vector for gene replacement in *C. glutamicum*. It was constructed by ligating a 2.6 kb *Pst*I DNA fragment containing *sacB*, the levansucrase gene of *Bacillus subtilis* (Schweizer 1992), to *Pst*I-digested pHSG299 (Takeshita et al. 1987), an *E. coli* vector carrying the kanamycin resistance gene. Plasmids pChom59, pClysC311, and pCpyc458, containing the mutated *hom*, *lysC*, and *pyc* genes, respectively, in vector pESB30 were used to replace the wild-type chromosomal genes with the mutated genes.

Media

Complete medium BY (Katsumata et al. 1984), minimal medium MM (Ozaki et al. 1985), and enriched minimal medium MMYE (Katsumata et al. 1984) were used for cultivation of *C. glutamicum*. Solid plates were made by the addition of Bacto-Agar (Difco) to 1.6%. When required, kanamycin and L-homoserine were added to final concentrations of 20 μg/ml for BY plates and 50 μg/ml for MM plates, respectively. LSS1 medium used for

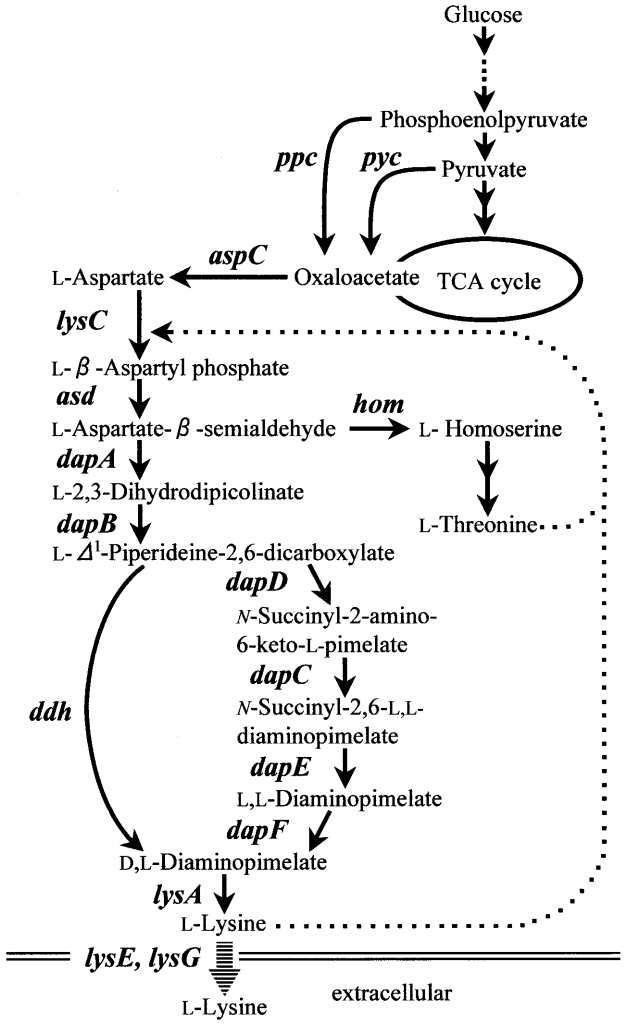

Fig. 2 Pathway and primary regulation of L-lysine biosynthesis in *Corynebacterium glutamicum*. The *broken arrow* indicates feedback inhibition. The enzymes encoded by the genes are as follows: *ppc* phosphoenolpyruvate carboxylase, *pyc* pyruvate carboxylase, *aspC* aspartate aminotransferase, *lysC* aspartokinase, *asd* aspartate semialdehyde dehydrogenase, *hom* homoserine dehydrogenase, *dapA* dihydrodipicolinate synthetase, *dapB* dihydrodipicolinate reductase, *dapD* tetrahydrodipicolinate succinylase, *dapC* succinyldiaminopimelate aminotransferase, *dapE* succinyl-L-diaminopimelate desuccinylase, *dapF* diaminopimelate epimerase, *lysA* diaminopimelate decarboxylase, *ddh* diaminopimelate dehydrogenase, *lysE* lysine exporter, *lysG* lysine export regulator

second-seed culture in jar fermentation consisted of (per liter) 50 g of sucrose, 40 g of corn steep liquor, 8.3 g of $(NH_4)_2SO_4$, 1 g of urea, 2 g of KH_2PO_4, 0.83 g of $MgSO_4 \cdot 7H_2O$, 10 mg of $FeSO_4 \cdot 7H_2O$, 1 mg of $CuSO_4 \cdot 5H_2O$, 10 mg of $ZnSO_4 \cdot 7H_2O$, 10 mg of β-alanine, 5 mg of nicotinic acid, 1.5 mg of thiamine-HCl, 0.5 mg of D-biotin, and 30 g of $CaCO_3$ (pH 7.2). LPG1 medium used for 5-l jar fermentors 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 $MgSO_4 \cdot 7H_2O$, 50 mg of $FeSO_4 \cdot 7H_2O$, 13 mg of $MnSO_4 \cdot 5H_2O$, 50 mg of $CaCl_2 \cdot 2H_2O$, 6.3 mg of $CuSO_4 \cdot 5H_2O$, 1.3 mg of $ZnSO_4 \cdot 7H_2O$, 5 mg of $NiCl_2 \cdot 6H_2O$, 1.3 mg of $CoCl_2 \cdot 6H_2O$, 1.3 mg of $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 23 mg of β-alanine, 14 mg of nicotinic acid, 7 mg of thiamine-HCl, and 0.5 mg of D-biotin (pH 7.0). For growth of *E. coli*, LB medium (Sambrook and Russell 2001) was used.

Table 1 PCR primer sequences used for amplification of biosynthetic genes from strain B-6

Target gene	Oligonucleotide sequence
ppc-F	5′-GCTGGCTGCCAACGCTGCGAGCGT-3′
ppc-R	5′-CCACCGAAGAGGCTGGAGAGTCCGC-3′
pyc-F	5′-CTGTGGCAGTGACCAACCGTCTGA-3′
pyc-R	5′-TCGTGTGCATGGTCCATCATGACAC-3′
aspC-F	5′-CTAATTGAGAGATTCTCGGTCATAT-3′
aspC-R	5′-ATCTCACTTCGCACATCGACCGTTTC-3′
lysC-asd-F	5′-GACAGGACAAGCACTGGTTGCACTA-3′
lysC-asd-R	5′-GTCGGTAGACCCAGTTTTTCCACTGA-3′
hom-F	5′-GCCACGCTCACTGCATATCGGAGACAT-3′
hom-R	5′-AGCCATCAAGAGGGACTTCGCCTTG-3′
dapA-F	5′-GCTTAATGCGCTGGAAGAAAAACT-3′
dapA-R	5′-TGGGAACCCTGAGAATCTTGGGATC-3′
dapB-F	5′-ACATATCGGAGTCACCAACTTGAGC-3′
dapB-R	5′-CATTGGAAGCAGTTCGAGGGTTCGGCT-3′
dapC-F	5′-ACGATGCGGTGTACGCAGCTTGTTC-3′
dapC-R	5′-GCCCGCGTCGAAGGAGAAGCGAGTAC-3′
dapD-F	5′-ATCCTGGTTTGAGTTCAGAGTTCAC-3′
dapD-R	5′-TGTTGTGCCAGCGGCATCACAATATG-3′
dapE-F	5′-ATGAGTCCGCGGGACCGCAGAGCAC-3′
dapE-R	5′-GATCGGCGTGCTTCCAATTGTGATCA-3′
dapF-F	5′-GAGCTGGGAGTCTGGTTCTCAGTCAT-3′
dapF-R	5′-CTATCGAGAAATTTCATTATGAAATC-3′
lysA-F	5′-CAACGAGGTGGTAGTTTTGGTACATG-3′
lysA-R	5′-TTCATACGACAATGACCGGCTTTTCG-3′
ddh-F	5′-GGCAGCGTATTCTCTGCAACTGGCA-3′
ddh-R	5′-AATCACCTCATCCGAGGCAAACCAGA-3′
lysE-lysG-F	5′-AGCAGTCAACCCACCTCGGGTTGGCT-3′
lysE-lysG-R	5′-TTGGCGACACCACCGATTTCACCG-3′

Recombinant DNA techniques

Standard protocols (Sambrook and Russell 2001) were used for the construction, purification and analysis of plasmid DNA, and transformation of *E. coli*. Chromosomal DNA was extracted from protoplasts of *C. glutamicum* ATCC 13032 and B-6 by the method of Saito and Miura (1963). The protoplasts were prepared by the method of Katsumata et al. (1984). Transformation of *C. glutamicum* by electroporation was carried out by the method of van der Rest et al. (1999), using a Gene Pulser and Pulse controller (Bio-Rad, USA). PCR was performed with a DNA Thermal Cycler GeneAmp 9600 (Perkin Elmer, USA), using *Taq* polymerase obtained from Boehringer Mannheim (Germany).

Nucleotide sequence analysis

A total of 16 genes (shown in Fig. 2) were amplified by PCR from genomic DNA of the L-lysine producer *C glutamicum* B-6. PCR primer sequences designed based on the nucleotide sequences of regions flanking each intact gene are shown in Table 1. The PCR products were cloned, after purification using a QIAquick PCR purification kit (Qiagen, Valencia, Calif.), into pESB30 using the TA cloning method (Sambrook and Russell 2001). The T vector was made by digesting pESB30 with *Bam*HI followed by blunting with DNA Blunting Kit (Takara Shuzo, Japan) and adding T to the blunt ends using *Taq* polymerase and dTTP. The nucleotide sequences were then analyzed in an ABI PRISM 377 DNA sequencer from Applied Biosystems using the dideoxy-DNA chain-termination method (Sanger 1981), with an ABI PRISM Big Dye Terminator Cycle Sequencing Kit (Perkin Elmer, USA). The subsequent electrophoresis analysis was carried out by Pageset SQC-5ALN 377 (Toyobo, Japan). That the mutations occurred in the genes, and were not artificially caused by *Taq* polymerase, was confirmed by analysing two independent clones. The recombinant plasmids constructed by cloning the PCR fragments containing the *hom*, *lysC*, and *pyc* genes from the *C. glutamicum* B-6 chromo-

some into pESB30 are designated pChom59, pClysC311, and pCpyc458, respectively, which were also used for the subsequent strain construction. The DNA sequences of *hom*, *lysC* and *pyc* have been submitted to GenBank and have been assigned the accession numbers AX123536, AX120365, and AX120849, respectively.

Generation of defined mutants HD-1, AK-1, AHD-2, and AHP-3

The *hom* and *lysC* mutations on pChom59 and pClysC311, respectively, were introduced into *C. glutamicum* ATCC 13032 via two recombination events. Since plasmids pChom59 and pClysC311 cannot replicate in *C. glutamicum*, transformation of *C. glutamicum* ATCC 13032 with each plasmid and subsequent selection for the plasmid marker kanamycin resistance yielded transformants which had integrated plasmid DNA into the genome via a single-crossover homologous recombination event. Next, each kanamycin-resistant integrant was grown without kanamycin for 1 day to allow a second recombination event to take place, and an appropriate dilution (10^4–10^5 cells) was spread on a BY plate containing 10% sucrose, which yielded about 10^2 colonies. The *sacB* positive selection system (Jäger et al. 1992) was used for this purpose. Since the expression of integrated plasmid-borne *sacB* in the presence of sucrose is lethal to *C. glutamicum*, only cells in which *sacB* is deleted as a consequence of the second homologous recombination can grow on the selective plate. In this recombination, allelic replacement arises when the wild-type *hom* or *lysC* gene is deleted from the genome, together with *sacB*. Ten randomly chosen sucrose-resistant colonies for each transformant were thus examined for the presence of the *hom* or *lysC* mutation by allele specific PCR (Kwok et al. 1995) to isolate the positive recombinant carrying the mutation. For this PCR analysis, the following site-specific primers were used:

- *lysC311*-F: 5′-GACGGCACCACCGACATCAT-3′
- *lysC311*-R: 5′-AGACCAGCGGCATCGTGAAGTGGCT-3′
- *hom59*-F: 5′-AGGTTCGTGGCATTGCTGC-3′
- *hom59*-R: 5′-CGGTTGGCGCGCCACCTGCACCG-TTTCCGT-3′

Underlined residues at the 3′ terminus represent the single-base mutations to discriminate mutants from wild-type sequences. The presence of the single base mutations was confirmed by DNA sequencing. Strains carrying the *hom* and *lysC* mutations in the wild-type background were designated HD-1 and AK-1, respectively. As with the construction of strain AK-1, plasmid pClysC311 was further used to introduce the *lysC* mutation into strain HD-1 to construct the *hom-lysC* double mutant AHD-2. Introduction of the *pyc* mutation into strain AHD-2 was carried out with the use of plasmid pCpyc458 as described above to construct the *hom-lysC-pyc* triple mutant AHP-3. For allele-specific PCR to verify the presence of the *pyc* mutation, the following site-specific primers were used:

- *pyc458*-F: 5′-CCGGATTCATTGCCGATCACT-3′
- *pyc458*-R: 5′-CTTGAGCTGCAGCAAAGTAG-3′

Enzyme assays

Crude cell extracts were prepared by sonic disruption of cells grown in MMYE medium as described previously (Ozaki et al. 1985). Protein quantity was determined by the method of Bradford (1976). Aspartokinase activities in crude cell extracts were measured essentially as described by Follettie et al. (1993).

L-Lysine production in 5-l jar fermentor cultures

Cells grown on a BY plate at 30°C for 1 day were inoculated into 250 ml of LSS1 medium in a 2-l flask. After cultivation at 30°C on a rotary shaker to early stationary phase, the seed broth was transferred into a 5-l jar fermentor containing 1,400 ml of LPG-1 medium. After the sugar initially added was consumed, a solution containing 50% (w/v) glucose, 4.5% (w/v) NH_4Cl, and 0.5 mg/l D-biotin was continuously fed until the total amount of sugar in the medium reached 25%. The feeding rate of the solution was controlled to maintain the glucose concentration in the medium at a low concentration (below 0.5%). The culture was performed with an agitation speed of 800 rpm, aeration at 2 l/min, and at 34°C. The pH was maintained at 7.3 with NH_4OH.

Analysis

Cell growth was monitored by measuring OD_{660} of the culture broth with a U-1080 Auto Sipper Photometer (Hitachi, Japan). Glucose concentration was determined using Determinar GL-E (Kyowa Medex, Japan). L-Lysine titer was determined as L-lysine HCl by HPLC (Shimazu, Japan) after derivatization with *o*-phthalaldehyde (Hill et al. 1979).

Results

Identification of mutation points by comparative genomic analysis

C. glutamicum B-6 (Hirao et al. 1989) is an L-lysine-hyperproducing strain constructed by an iterative procedure of random mutation and selection from the wild-type strain ATCC 13032. This strain certainly has the ability to produce high levels of L-lysine from inexpensive carbon and nitrogen sources. However, it produces the amino acid inefficiently due to poor growth and sugar consumption, similar to other L-lysine-producing strains reported (Leuchtenberger 1996; Oka 1999), thus leading to the suboptimal production rate of less than 2.0 g l^{-1} h^{-1}.

Our initial task was to identify mutations on the relevant terminal pathways, the efflux step, and the anaplerotic reactions. For this purpose, a total of 16 genes indicated by the gene symbols in Fig. 2, covering each putative promoter and terminator regions, were isolated from genomic DNA of strain B-6 by PCR, and their nucleotide sequences were determined. The sequences were then compared with the corresponding wild-type sequences, revealing that the *hom*, *lysC*, *dapE*, *dapF*, and *pyc* genes each had a point mutation in the coding region: a T to C exchange at position 176 in *hom*, leading to amino acid replacement of Val-59 by Ala; a C to T exchange at position 932 in *lysC*, leading to replacement of Thr-311 with Ile; a C to T exchange at position 771 in *dapE*; a C to T exchange at position 582 in *dapF*; and a C to T exchange at position 1372 in *pyc*, leading to replacement of Pro-458 with Ser. No mutation was observed in the other 11 genes including the efflux genes *lysE* and *lysG*, indicating that they remained wild-type in strain B-6. Among the five single base-pair mutations identified, we considered the *dapE* and *dapF* mutations negligible because each mutation resulted in neither amino acid substitution nor change to any rare codons. Thus, we limited our attention to the *hom* mutation (V59A, designated *hom59*), the *lysC* mutation (T311I, designated *lysC311*), and the *pyc* mutation (P458S, designated

pyc458). None of these specific mutations have been reported previously and we next examined whether they are relevant to L-lysine production.

Evaluation of *hom59* and *lysC311*

It is known that a mutation resulting in the defect of homoserine dehydrogenase, the *hom* product, or desensitization of aspartokinase, the *lysC* product, leads to elimination of the bottleneck in the biosynthetic pathway from L-aspartic acid toward L-lysine, thereby allowing L-lysine production in *C. glutamicum* and related species (Shiio and Miyajima 1969; Sano and Shiio 1971; Kase and Nakayama 1974). If this is the case with *hom59* or *lysC311*, it is expected that the presence of the mutation in the wild-type genome would result in L-lysine accumulation. To examine this possibility, each mutation was introduced into *C. glutamicum* wild-type strain ATCC 13032 by two-step homologous recombination using plasmids pChom59 and pClysC311 containing the mutated *hom* and *lysC* genes from strain B-6, respectively, as described in Materials and methods. DNA sequencing confirmed that the resulting mutants, designated HD-1 and AK-1, carried the *hom59* and *lysC311* mutations, respectively, in the wild-type background. Growth experiments revealed that strain HD-1 required L-homoserine for maximum growth on MM plates, suggesting that *hom59* was a leaky mutation leading to a partial requirement for L-homoserine for growth. Likewise, strain AK-1 exhibited the expected phenotype of resistance to AEC, an L-lysine structural analog, indicating that *lysC311* was an AEC-resistant mutation and was responsible for the AEC resistance of the original strain B-6. Assays using crude extracts of strain AK-1 showed that the mutated aspartokinase was indeed desensitized to synergistic inhibition by L-lysine plus L-threonine. When L-lysine and L-threonine were added at 10 mM each to the assay, the mutated aspartokinase retained near 100% of its activity while the wild-type enzyme from strain ATCC 13032 retained only about 5% of its activity. However, in the presence of a higher concentration of L-lysine (100 mM) together with L-threonine (10 mM), the activity of the mutated aspartokinase was inhibited by about 50%, revealing incomplete deregulation of the mutated enzyme.

L-Lysine production by strains HD-1 and AK-1 was investigated using 5-l jar fermentors, with wild-type ATCC 13032 and classically derived B-6 as controls. Figure 3 shows the profiles obtained in fed-batch fermentor cultivation with glucose medium to which sugar was added at an initial concentration of 5%, with a subsequent increase to 25%. As indicated, the slow-growing B-6 required more than 50 h to complete fermentation at around 95 g/l of L-lysine. In contrast, strains HD-1 and AK-1 grew and consumed glucose as fast as the wild-type strain, thus leading to completion of fermentations within 30 h. In the case of strain AK-1, the final growth level was somewhat limited as can be seen from the OD. In these fermentations, strains HD-1 and AK-1 accumulated 8 g and 55 g of L-lysine/l, respectively, showing that both mutations were relevant to production.

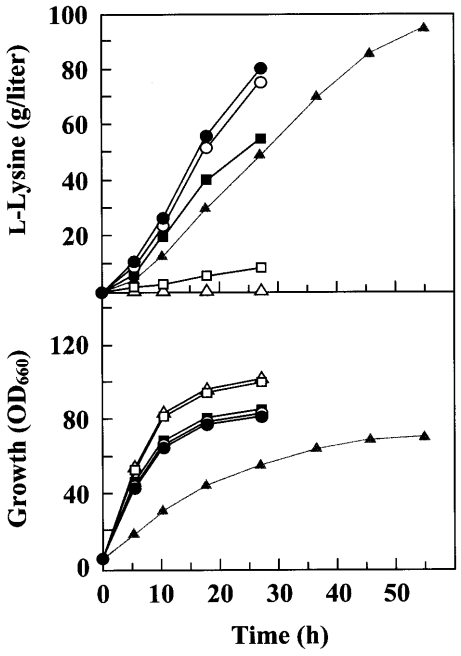

Fig. 3 L-Lysine fermentation by strains HD-1 (*open squares*), AK-1 (*black squares*), AHD-2 (*open circles*), and AHP-3 (*black circles*) in fed-batch fermentor cultivation. For comparison, the profiles of the wild-type strain ATCC 13032 (*open triangles*) and the classically derived L-lysine-producing strain B-6 (*black triangles*) are shown as controls. Data represent mean values from three independent cultures

Reconstitution of *hom59* and *lysC311*

Based on our methodology for strain reconstruction, we combined the beneficial *hom59* and *lysC311* mutations by introducing *lysC311* into strain HD-1 (*hom59*) to construct strain AHD-2, carrying both the *hom* and *lysC* mutations on its genome. Growth experiments revealed that strain AHD-2 no longer required L-homoserine for maximum growth and that it exhibited almost the same growth rate on a MM plate as the wild-type strain. The suppression of the *hom59*-induced phenotype of the partial requirement for L-homoserine by the coexistence with *lysC311* would occur because the mutated homoserine dehydrogenase of strain AHD-2 might proceed better with the reaction when the substrate L-aspartate-β-semialdehyde was oversupplied through AEC-resistant aspartokinase activity.

Strain AHD-2 was then tested for L-lysine production in 5-l jar fermentors (Fig. 3). This strain showed almost the same growth profile as strain AK-1 and produced much more L-lysine (75 g/l) than strain AK-1, indicating that both the *hom* and *lysC* mutations exhibited a synergistic effect on production.

Introduction and evaluation of *pyc458*

Following the removal of the bottleneck in the terminal pathway, we shifted our target to the anaplerotic pathways in central metabolism and attempted to evaluate the *pyc458* mutation in strain AHD-2. The *pyc* mutation was thus introduced into strain AHD-2 by allelic replacement using plasmid pCpyc458, resulting in the creation of strain AHP-3 that carried the *pyc* mutation in addition to the *hom* and *lysC* mutations in its genome. Comparative phenotypic analyses showed no distinct differences between strains AHD-2 and AHP-3 with respect to the drug-resistant phenotypes used as selection markers for isolation of strain B-6 from the wild-type strain ATCC 13032 (see Materials and methods), indicating that the *pyc458* mutation was not related to any drug resistance in strain B-6.

L-Lysine production by strain AHP-3 was then investigated using jar fermentors. As shown in Fig. 3, this *hom-lysC-pyc* triple mutant grew and consumed glucose as fast as strain AHD-2 and produced more L-lysine than the *hom-lysC* double mutant. Three independent cultures showed that the effect was reproducible and significant, indicating that *pyc458* is a beneficial mutation for L-lysine production. Accumulation of as much as 80 g of L-lysine/l after only 27 h by strain AHP-3 indicated that reconstitution of just these three point mutations in the wild-type genome resulted in the highest production rate (3.0 g l^{-1} h^{-1}) among fed-batch fermentations of L-lysine reported to date. As a result, the fermentation period was shortened to nearly half of that traditionally required (Oka 1999).

Discussion

We demonstrate in this study that reconstruction of classically derived production strains based on genomic information can be an effective approach for innovation of fermentation processes in the post-genomic era. The key aspect of this approach is to enable a production strain to make full use of its intrinsic ability through eliminating all undesirable mutations accumulated in its genome, thereby leading to, for example, substantial shortening of fermentation times. Such high-speed fermentation, as demonstrated here, is a significant breakthrough, especially in manufacture of large-scale bulks such as L-lysine, because it allows remarkable increases in overall productivity as well as production capacity without the need for new fermentors, which not only reduces production costs but also minimizes capital investment.

In addition to the practical advantages, the process of strain reconstruction avoids the complication of uncharacterized secondary mutations and contributes to rationalizing the mechanism of production through characterization of the genetic background for high-level production. The effects of mutations *hom59* and *lysC311* on L-lysine production are reasonably comprehensible based on well-established knowledge (Shiio and Miyajima 1969; Sano and Shiio 1971; Kase and Nakayama 1974). However, that the two mutations exerted a synergistic effect on production upon their coexistence in the wild-type genome was unexpected as they were regarded as the alternatives to L-lysine production. Presumably, the *lysC311* mutation which leads to partial deregulation of aspartokinase by L-lysine plus L-threonine could achieve higher deregulation of the enzyme with the help of the *hom59* mutation leading to the limitation of the intracellular pool of L-threonine. This postulated cooperation is also the fundamental mechanism of L-lysine production in the classically derived producer B-6.

The discovery of the beneficial *pyc* mutation has also illustrated the usefulness of the genome-based approach. While importance of the anaplerotic enzyme pyruvate carboxylase for L-lysine production has been demonstrated by the study with a *pyc*-overexpressing strain of *C. glutamicum* (Peters-Wendisch et al. 2001), no *pyc* mutations leading to increased production of amino acids have been reported. Therefore, such an unexpected mutation, with no known selectable phenotype, would be almost impossible to find without the use of comparative genomic information. The *pyc458* mutation (P458S) is located outside of any motifs involved in ATP-binding, pyruvate-binding, and biotin-binding, and thus seems not to be directly involved in substrate binding. Since proline is known to have the lowest conformational flexibility of all the amino acids (Branden and Tooze 1991), its replacement by serine might permit a relief in conformational constraint and contribute to improved catalytic ability under the fermentation conditions used. Verification of this must await studies on the catalytic properties of this mutant enzyme and also on its structure-function relationships.

The genome-based approach presented here is providing important insights into the production capabilities inherently possessed by *C. glutamicum*. One intriguing finding involves L-lysine export, which has been discussed in relation to L-lysine overproduction (Vrljic et al. 1996; Hua et al. 2000). The export process is catalyzed by a recently discovered transport carrier, the product of *lysE* (Vrljic et al. 1996), the synthesis of which is controlled by the regulator protein encoded by *lysG*. Interestingly, our comparative genomic analysis revealed no mutations in either *lysE* or *lysG* of strain B-6, indicating that *C. glutamicum* is naturally endowed with a high capability to excrete L-lysine. This is also supported by the fact that introduction of only *hom59*, *lysC311*, and *pyc458* into the wild-type strain achieved, to our knowledge, the highest rate of L-lysine production to date. This prominent characteristic of L-lysine excretion may be the reason why this bacterium has been widely used for the industrial fermentation of L-lysine.

Our approach allows the construction of a defined mutant without any foreign DNA, and thus is desirable for industrial uses. Toward the goal of industrial contribution, we have been now shifting our target from the downstream pathways to upstream in central metabo-

lism. Although this strain reconstruction program is ongoing, the present results have obviously demonstrated the potential of this approach to significantly extend the performance of existing production strains and also exemplified how traditional fermentation can benefit from the influx of genomic data.

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