Lysine Fermentation – History and Genome Breeding

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Abstract

Lysine fermentation by *Corynebacterium glutamicum* was developed in 1958 by Kyowa Hakko Kogyo Co. Ltd. (current Kyowa Hakko Bio Co. Ltd.) and is the second oldest amino acid fermentation process after glutamate fermentation. The fundamental mechanism of lysine production, discovered in the early stages of the process's history, gave birth to the concept known as "metabolic regulatory fermentation", which is now widely applied to metabolite production. After the development of rational metabolic engineering, research on lysine production first highlighted the need for engineering of the central metabolism from the viewpoints of precursor supply and NADPH regeneration. Furthermore, the existence of active export systems for amino acids was first demonstrated for lysine in *C. glutamicum*, and this discovery has resulted in the current recognition of such exporters as an important consideration in metabolite production. Lysine fermentation is also notable as the first process to which genomics was successfully applied to improve amino acid production. The first global "genome breeding" strategy was developed using a lysine producer as a model; this has since led to new lysine producers that are more efficient than classical industrial producers. These advances in strain development technology, combined with recent systems-level approaches, have almost achieved the optimization of entire cellular systems as cell factories for lysine production. In parallel, the continuous improvement of the process has resulted not only in fermentation processes with reduced load on downstream processing, but also in commercialization of various product forms according to their intended uses. Nowadays lysine fermentation underpins a giant lysine demand of more than 2 million metric tons per year.

Keywords

Lysine market, Lysine production technology, Downstream processing, Product forms, Lysine-producing strains, *Corynebacterium glutamicum*, *Escherichia coli*, Genome breeding, Systems metabolic engineering

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Introduction

Lysine, one of the essential amino acids for animals, has a significant commercial value as a feed additive to promote the growth of animals including swine and poultry, and thus is the second-ranking amino acid after glutamate with regard to worldwide annual production. Lysine is also used as a fish feed additive, because lysine is generally the first limiting essential amino acid in many protein sources used in fish feeds (Hua 2013). The global lysine market is currently estimated to be 2.2 million metric tons per year (Ajinomoto 2013; Eggeling and Bott 2015), and is still growing at annual rates of around 10% (Fig. 5.1). As the scale of production has increased, lysine prices per kilogram have dropped to around 1.5 USD, fluctuating between 1.3 and 2.5 USD over the past decade (Ajinomoto 2013; Eggeling and Bott 2015), depending largely on competition from natural lysine sources such as soybean meal and sardine (Fig. 5.1). Because only the L-form of lysine is effective as a feed additive, this amino acid is manufactured through fermentation. The main suppliers are CJ CheilJedang (South Korea), Global Bio-chem Technology Group (China), Ajinomoto (Japan), Archer Daniels Midland (USA), and Evonik Industries (Germany), among others (Ajinomoto 2013; Eggeling and Bott 2015). Major commercial plants are located in the respective corn belts in China, North America, Brazil, Indonesia, and Russia.

The history of lysine fermentation goes back to the late 1950s when Kyowa Hakko Kogyo (current Kyowa Hakko Bio) found that a homoserine-auxotrophic mutant of

Corynebacterium glutamicum produced significant amounts of lysine in liquid medium (Nakayama et al. 1961). Based on the patents issued at that time, the first-generation lysine producers with homoserine auxotrophy seem to have been capable of achieving final titers of 40-60 g/L and around 25% fermentation yields (w/w) of lysine hydrochloride from sugar (Kitada et al. 1961; Kyowa Hakko Kogyo 1970). Further improvement was persistently carried out in the 1970s and 1980s by inducing mutants with additional amino acid auxotrophies and resistance to antimetabolites including the lysine analogue *S*-(2-aminoethy)-L-cysteine (AEC). The titers produced by these strains reached 100 g/L with yields of 40-50% on sugar in fed-batch cultures (Leuchtenberger 1996; Ikeda 2003). In the late 1980s and 1990s, various tools for genetic engineering of this microbe were exploited, and these molecular techniques were applied to strain improvement aimed at enhancing lysine production (Pfefferle et al. 2003; Kelle et al. 2005). This allowed rational metabolic engineering not only for the lysine-biosynthetic pathways but also for the central metabolism: more carbon could be directed toward the terminal pathways and the NADPH supply could be augmented. Since the beginning of the 2000s, systems-level approaches, including in-silico modeling and simulation approaches, have come to be used to help identify new targets for further strain improvement (Krömer et al. 2004; Wendisch et al. 2006; Wittmann and Becker 2007; Kjeldsen and Nielsen 2009). Meanwhile, the availability of high-throughput DNA sequencing has made it feasible to decode the genomes of classical industrial producers and thereby to identify important genetic traits that distinguish them from their wild-type ancestors. As a result, the conventional style of selecting improved strains by their phenotypes, formerly the standard practice in the industry, is rapidly being replaced by a new method called “genome breeding”, where desirable genotypes are systematically assembled in a wild-type genome (Ikeda et al. 2006; Lee et al. 2012; Kim et al. 2013). The reconstructed strains can be more robust, give

higher fermentation yields in less time, and resist stressful conditions better than classical industrial producers.

According to recent publications (Kelle et al. 2005; Eggeling and Bott 2015), the current yields on sugar and titers of lysine hydrochloride can be estimated at 55-60% and 120-170 g/L, respectively. This means that strain development for lysine production almost achieves the optimization of entire cellular systems. For a half century following the start of lysine fermentation, *C. glutamicum* and its relatives were the sole production organism, but recently, *Escherichia coli* has also come to play a significant role in the process of lysine fermentation (Nagai et al. 2009; Ying et al. 2014; He et al. 2015).

This chapter first briefly describes the general outlines of lysine fermentation and typical production strains, then highlights advances in lysine production technology with a special focus on genome breeding methodology, which has currently become a standard practice in the amino acid industry. The biosynthesis of lysine, including the relevant pathways, enzymes, and genes, has been omitted because such fundamental information can be found in many other publications (Pfefferle et al. 2003; Kelle et al. 2005; Wittmann and Becker 2007).

2

Fermentation Processes of Lysine

2.1

Fermentation Operations

Industrial fermentation processes typically comprise two steps: cultivation of a strain for lysine production and downstream processing including purification of lysine from fermented broth and wastewater treatment. The lysine industry is highly competitive in the world market, and the

entire manufacturing process has so far been optimized from various perspectives including the cost of the raw materials, the fermentation yield, the purification yield, the productivity of the overall process, the investment cost, the cost of waste-liquor treatment, the formulation of the product, and so on. Since the carbon source is the major cost factor in the fermentation process, its selection is of primary importance. As the main source of carbon, cane molasses, beet molasses, sucrose, and starch hydrolysates (glucose or dextrose) from corn, cassava, and wheat are widely used in industrial processes. The preferred carbon source among these varies from one region to another. For example, starch hydrolysate from corn, i.e., corn syrup, is the usual carbon source in North America, China, and Indonesia, while cane and beet molasses are advantageously used in Europe and South America, respectively, on the basis of each substance's cost and availability in these regions.

Industrial lysine fermentation is usually performed by means of fed-batch processes using large-scale tank fermenters in the size range of 500 kl or larger. In production plants where the fed-batch process is used, lysine accumulates to a final titer of 170 g/L after 45 h (Eggeling and Sahm 1999). To improve overall productivity further, it is possible to extend fed-batch fermentation by drawing out part of the broth one or more times during the process and refilling it through nutrient feeding (semi-continuous fermentation), or by shifting the fermentation from batch to continuous culture, in which fresh medium containing all nutrients is fed into a fermenter at a specific rate while the same quantity of broth with a portion of the microorganisms is continuously taken from the fermenter, thus maintaining a constant culture volume (continuous fermentation). In continuous fermentation, stable lysine production for more than 300 h was demonstrated with a *C. glutamicum* lysine-producing mutant; this process yielded a maximum volumetric productivity value of 5.6 g/L per hour, which is more than 2.5 times higher than that seen in fed-batch culture with the same strain (Hirao et al. 1989).

Industrial processes for recovering lysine from the fermented broth differ depending on many factors such as the grade and intended use of the product, the raw materials used, local environmental regulations, and so on. In the past, recovery of feed-grade lysine from the broth has mainly depended on the conventional chromatographic method (Ikeda 2003). In this method, after the cell mass was removed through centrifugation or ultrafiltration, lysine was purified as a hydrochloride salt using an ion exchange resin; this was followed by crystallization or spray-drying. Chromatographic purification can result in a higher quality product, but has the disadvantage of generating lower product concentrations and larger volumes of waste liquor, increasing the cost of waste-liquor treatment. To cope with this problem, organic compound fertilizers were manufactured as a means of effectively utilizing the waste liquor from lysine fermentation, recycling resources and protecting the environment (Ikeda 2003).

Recently, however, downstream processes for feed-grade lysine have become much simpler and more economical. After removal of the cell mass, the filtrate is merely evaporated and, in some cases, spray-dried. Today, development to meet various requirements has resulted in commercialization of different product forms at lower prices (Kelle et al. 2005). These include liquid concentrates and granulated solids with different concentrations of lysine. An example of a liquid product is Liquid Lysine 60 (Feed Grade) from Ajinomoto Co. Inc., which contains free lysine and lysine hydrochloride at a concentration of 60%. An example of a granulate product is sold as Biolys® by Evonik Industries. This product is extracted directly from the fermented broth by evaporation and granulation without removal of the cell mass, and thus contains co-products such as other amino acids and proteins in addition to lysine (more than 54.6% purity as a sulfate salt).

A unique production process has been developed with the goal of making downstream processing more efficient (Kobayashi et al. 2011). While conventional processes utilize sulfate

and/or chloride as counter anion(s) to produce lysine sulfate and/or lysine hydrochloride, the new process predominantly utilizes hydrocarbonate and carbonate ions to produce lysine carbonate. This type of fermentation can be achieved by gradually shifting the pH of the culture to alkaline conditions where relatively high concentrations of hydrocarbonate and carbonate ions can exist. Since the hydrocarbonate and carbonate ions are derived from carbon dioxide gas, which itself is produced by a production strain, the amounts of ammonium sulfate and ammonium chloride added to the medium can be reduced, which allows for significant cost savings as well as reduction of the environmental loads resulting from downstream processing. This process also allows for the simple recovery of a high lysine content because the counter anions are easily discharged as carbon dioxide gas just by heating.

2.2

Production Strains

The industrial lysine producers used in the early stages of the process's history were regulatory mutants derived from *Corynebacterium glutamicum* and its subspecies, *flavum* and *lactofermentum*. The following two properties are considered to be most crucial for lysine production by these mutant strains: (i) homoserine auxotrophy due to a defect of homoserine dehydrogenase, and (ii) AEC resistance due to the desensitization of aspartokinase from feedback inhibition (Shiio and Miyajima 1969; Sano and Shiio 1971; Kase and Nakayama 1974; Pfefferle et al. 2003). Practical industrial strains were constructed by combining these two properties, resulting in lysine production with a conversion yield of approximately 30% on sugar (w/w) as lysine hydrochloride (Nakayama and Araki 1973; Kinoshita and Nakayama 1978).

In addition to these fundamental properties, leucine auxotrophy is known to increase lysine production (Tosaka et al. 1978; Patek et al. 1994; Schrumpf et al. 1992). This was originally explained as occurring due to release from the leucine-dependent repression of the *dapA* gene encoding dihydrodipicolinate synthase, which is the first enzyme of the lysine-biosynthetic pathway (Tosaka et al. 1978). More recently, however, since no significant up-regulation of *dapA* was observed under leucine-limited conditions, it is believed that a different mechanism is likely to be involved in the positive effect of leucine limitation on lysine production (Hayashi et al. 2006a). In relation to this, it has been reported that leucine limitation gives rise to the global induction of the amino acid biosynthesis genes, including the *lysC* gene encoding the key enzyme aspartokinase (Hayashi et al. 2006a).

Further strain development was carried out introducing auxotrophy for alanine or vitamins, resistance to antimetabolites such as 6-azauracil, naphtoquinoline, 3, 3', 5-L-triiodo-L-thyronine, α-chlorocaprolactam, and $N^{\alpha}N^{\varepsilon}$-dioctanoyl-L-lysine, and resistance to antibiotics such as rifampicin and streptomycin (Ikeda 2003; Kelle et al. 2005). Additional screening and selection for these phenotypic characters has resulted in lysine production with yields of 40-50% on sugar (Enei et al. 1989).

Since lysine requires oxaloacetate and pyruvate as precursors for its biosynthesis, flux balances leading to optimal precursor supply are crucial for efficient lysine production. From this perspective, production strains were further improved by means of classical mutagenesis, which has led to strains capable of producing lysine with conversion yields of up to 50% on sugar. These include pyruvate kinase-deficient mutants and β-fluoropyruvate-sensitive mutants with decreased pyruvate dehydrogenase activities (Ozaki and Shiio 1983; Shiio et al. 1984; Shiio and Nakamori 1989). Defect or reduction of phosphoenolpyruvate carboxykinase (Petersen et al. 2001; Riedel et al. 2001), isocitrate dehydrogenase (Becker et al. 2009), or citrate synthase (Shiio

et al. 1982; Yokota and Shiio 1988) was used as another option for the same objective of increasing availability of the precursors.

In addition to *C. glutamicum* strains, *E. coli* strains have been found to be useful in industrial processes (Nagai et al. 2009). Lysine-producing strains of *E. coli* have been developed mainly by rational approaches. These include amplification and deregulation of the rate-limiting enzymes on the terminal pathway (Kojima et al. 1995; Ogawa-Miyata et al. 2001), deletion of lysine-degradation pathways (Kikuchi et al. 1997), amplification of the lysine exporter YbjE (Ueda et al. 2005; Pathania and Sardesai 2015), enhancement of respiratory chain pathways with high energy efficiency or reduction of those with low energy efficiency (Nakai et al. 2002), disruption of the ribosome modulation factor (Imaizumi et al. 2005), and overexpression of ppGpp synthetase (Imaizumi et al. 2006). The lysine fermentation performance of such *E. coli* strains has almost reached a level comparable to that of *C. glutamicum* strains. More than 130 g/L of lysine production with a yield of 45% on glucose has been demonstrated by using a threonine and methionine double-auxotrophic *E. coli* strain carrying a plasmid which contains the *ppc* gene encoding phosphoenolpyruvate carboxylase, the *pntB* gene encoding pyridine nucleotide transhydrogenase, and the *aspA* gene encoding aspartate ammonia-lyase (Ying et al. 2014). *E. coli* generally shows faster growth at higher temperatures than *C. glutamicum* does, which is why *E. coli* strains may be economically advantageous, especially in tropical regions, because in those regions the cost of utilities is not negligible to keep the fermentation temperature optimum.

Thermotolerant bacterial species such as *Corynebacterium efficiens* and *Bacillus methanolicus* have also drawn attention as promising lysine producers (Nagai et al. 2009). *C. efficiens*, formerly named *Corynebacterium thermoaminogenes*, is phylogenetically close to *C. glutamicum* but can grow at temperatures approximately 10ºC higher (Murakami et al. 1993; Fudou et al. 2002; Nishio et al. 2003). *B. methanolicus* is a thermotolerant methylotroph that

grows at 35-60ºC on methanol, an alternative carbon source which does not compete with human food (Lee et al. 1996).

The typical production strains, especially the regulatory mutants developed by classical mutagenic approaches, are listed in Table 5.1.

3

Metabolic Engineering for Lysine Production

Needless to say, lysine-producing mutant strains constructed according to the techniques described above were further improved through the use of recombinant DNA technology, although the practical applicability of such recombinant strains to industrial fermentation has not been disclosed. The targets of metabolic engineering have expanded beyond the core biosynthetic pathways leading to lysine and include central metabolism, cofactor-regeneration systems, uptake and export systems, energy metabolism, global regulation, and feedstock utilization (Fig. 5.2). In this section, recent advances in molecular strain improvement for lysine production are described.

3.1

Precursor Supply

As mentioned above, classical approaches aiming at increasing the supply of precursors into the lysine-biosynthetic pathway have led to incremental gains in lysine production. The same objective has also been achieved through molecular approaches. For example, carbon flux from glycolysis intermediates to oxaloacetate has been increased by overexpression of the pyruvate carboxylase gene (Peters-Wendisch et al. 2001), by deregulation of phosphoenolpyruvate

carboxylase (Chen et al. 2014), or by deletion of the phosphoenolpyruvate carboxykinase gene (Petersen et al. 2001; Riedel et al. 2001), resulting in significantly increased production of lysine. Increasing the availability of pyruvate by abolishing pyruvate dehydrogenase activity can also improve lysine production (Blombach et al. 2007). On the other hand, inactivation or attenuation of the TCA cycle enzyme(s) isocitrate dehydrogenase (Becker et al. 2009), citrate and methylcitrate synthases (Radmacher and Eggeling 2007, van Ooyen et al. 2012), or malate:quinone oxidoreductase (Mitsuhashi et al. 2006) was shown to improve lysine production, probably by means of a flux shift from the TCA cycle toward the lysine-biosynthetic pathway.

3.2

NADPH Availability

Availability of NADPH is crucial, especially for the production of lysine, because 4 mol of NADPH are required for the biosynthesis of 1 mol of lysine from oxaloacetate. In *C. glutamicum*, the usual strategy for increasing NADPH supply is the redirection of carbon from glycolysis into the pentose phosphate pathway, through disruption of the phosphoglucose isomerase gene (Marx et al. 2003) or through overexpression of the fructose 1,6-bisphosphatase gene (Becker et al. 2005) or the glucose 6-phosphate dehydrogenase gene (Becker et al. 2007). The introduction of a mutant allele of the 6-phosphogluconate dehydrogenase gene encoding an enzyme that is less sensitive to feedback inhibition can be another option (Ohnishi et al. 2005). In terms of carbon yield, however, supplying carbon through the pentose phosphate pathway is less advantageous than supplying it via the glycolytic pathway because the former pathway inevitably involves the release of 1 mol of carbon dioxide (CO_2) accompanied by the oxidation of 1 mol of hexose. To solve this dilemma, an attempt was recently made to engineer a functional glycolytic pathway in

C. glutamicum supplying NADPH through a new route (Takeno et al. 2010, 2016). In this study, endogenous NAD-dependent glyceraldehyde 3-phosphate dehydrogenase (GapA) of *C. glutamicum* was replaced with nonphosphorylating NADP-dependent glyceraldehyde 3-phosphate dehydrogenase (GapN) of *Streptococcus mutans*, leading to a *C. glutamicum* strain with an NADPH-generating glycolytic pathway (Fig. 5.3). A lysine producer derived from the engineered GapN strain has been shown to produce considerably more lysine than the reference GapA strain. Moreover, it has been demonstrated that blockade of the oxidative pentose phosphate pathway through a defect in glucose 6-phosphate dehydrogenase did not significantly affect lysine production in the engineered strain, while a drastic decrease in lysine production was observed for the reference strain (Takeno et al. 2016). Considering that the intracellular $NADPH/NADP^+$ ratio in the engineered strain was significantly higher than that of the reference strain irrespective of the pentose phosphate pathway, it seems reasonable to consider that the high $NADPH/NADP^+$ ratio not only allows cells to bypass the pentose phosphate pathway but also causes a decreased flux through the TCA cycle, resulting in increased availability of oxaloacetate and pyruvate for lysine biosynthesis. This study is the first to demonstrate efficient lysine production independent of the oxidative pentose phosphate pathway.

As alternatives to the *S. mutans* GapN enzyme, the *Clostridium acetobutylicum* GapN (Komati Reddy et al. 2015) and the modified endogenous GapA with preference of NADP (Bommareddy et al. 2014) have been successfully used for improved lysine production by *C. glutamicum*. On the other hand, expression of the membrane-bound transhydrogenase genes from *E. coli* in *C. glutamicum* has been shown to provide another source of NADPH (Kabus et al. 2007a).

3.3

Lysine Export

The export step is critical for achieving efficient amino acid production in *C. glutamicum* (Burkovski and Krämer 2002). The amino acid exporter first identified in bacteria is LysE, which exports the basic amino acids lysine and arginine in *C. glutamicum* (Vrljić et al. 1996). Overexpression of the *lysE* gene resulted in a five-fold increase in the excretion rate for lysine compared to the rate of the control strain (Vrljić et al. 1996). The functions of LysE also can be transferred to heterologous bacterial species. For example, a mutant allele of the *C. glutamicum lysE* gene has been successfully used to improve lysine production in the methylotroph *Methylophilus methylotrophus* (Gunji and Yasueda 2006). In *E. coli*, YbjE has been identified as a possible lysine exporter. The *ybjE* gene has been shown to confer AEC resistance and increased lysine production on *E. coli* strains when amplified on a multi-copy vector (Ueda et al. 2005; Nagai et al. 2009).

3.4

Glucose Uptake

In *C. glutamicum,* the phosphoenolpyruvate-dependent sugar phosphotransferase system (PTS) had long been the only known system to take up glucose, but recently, potential glucose uptake systems that function as alternatives to the PTS have been identified in this bacterium (Ikeda et al. 2011, 2013; Lindner et al. 2011a, b; Ikeda 2012). These include the *iolT1* and *iolT2* gene products, both known as *myo*-inositol transporters (Krings et al. 2006). Expression of the *iolT1*-specific glucose uptake bypass instead of the native PTS resulted in approximately 20% increased lysine production (Ikeda et al. 2011). This positive effect has been explained as

occurring through an increased availability of PEP, which contributes to an increased supply of carbon from the central metabolism into the lysine-biosynthetic pathway through an anaplerotic reaction involving PEP carboxylase (Fig. 5.4). More recently, a third non-PTS glucose uptake route consisting of the *bglF*-specified EII permease and native glucokinases has been identified in *C. glutamicum* ATCC 31833 (Ikeda et al. 2015). This will expand the options for the development of more efficient production strains.

3.5

Energy Efficiency

Like redox balance, energy balance is critical for efficient lysine production. For this reason, improving the efficiency of ATP synthesis is another strategy for increasing lysine production. In *C. glutamicum*, two terminal oxidases are positioned in a branched respiratory chain (Bott and Niebisch 2003). One branch is composed of the cytochrome bc_1-aa_3 supercomplex, which has a threefold-higher bioenergetic efficiency than the other cytochrome *bd* branch. Disruption of the inefficient cytochrome *bd* branch caused increased lysine production with no marked effect on growth or glucose consumption (Kabus et al. 2007b). The usefulness of this alteration of the respiratory chain pathway has also been demonstrated for *E. coli* lysine producers (Nakai et al. 2002).

Recently, it has been shown that *C. glutamicum* can grow anaerobically by means of nitrate respiration (Nishimura et al. 2007; Takeno et al. 2007). In the presence of nitrate, lysine and arginine production occurred anaerobically, though at a very low level, indicating the potential of this bacterium for anaerobic amino acid production (Takeno et al. 2007).

3.6

Global Regulation

Since amino acid biosynthesis in *C. glutamicum* is directly or indirectly subject to both pathway-specific and global regulation (Brockmann-Gretza and Kalinowski 2006; Krömer et al. 2008), global regulation is also important in strain improvement. Actually, it has been demonstrated through transcriptome analysis that the global induction of amino acid biosynthesis genes occurred in a classically-derived industrial lysine-producing strain of *C. glutamicum* (Hayashi et al. 2006b). In this strain, the *lysC* gene, encoding the key enzyme aspartokinase, was up-regulated several folds, though a repression mechanism for lysine biosynthesis is not known in *C. glutamicum*. Although the genetic elements responsible for these changes have not yet been identified, the introduction of a mutant allele of the *leuC* gene into a defined lysine producer has been shown to trigger a stringent-like global response and thereby to enable a significant increase in lysine production (Hayashi et al. 2006a).

3.7

Carbon Substrate Spectrum

The main feedstocks for industrial lysine fermentation by *C. glutamicum* are sugars from agricultural crops, such as cane molasses, beet molasses, and starch hydrolysates (glucose or dextrose) from corn and cassava. However, due to environmental concerns, considerable efforts have been made to broaden the substrate spectrum of this organism towards alternative raw materials, especially those that do not compete with human food or energy sources. For example, wild type *C. glutamicum* cannot utilize whey, which contains lactose and galactose, but

heterologous expression of both *lacYZ* from *Lactobacillus delbrueckii* subsp. *bulgaricus* and *galMKTE* from *Lactococcus lactis* subsp. *cremoris* in a *C. glutamicum* lysine producer has resulted in a strain that is able to produce lysine at up to 2 g/L from whey (Barrett et al. 2004). *C. glutamicum* is also unable to use starch, but expression of the α-amylase gene from *Streptomyces griseus* has allowed *C. glutamicum* to utilize soluble starch for lysine production, albeit at an efficiency lower than that obtained using glucose (Seibold et al. 2006). More efficient lysine production from soluble starch has been achieved in *C. glutamicum* by displaying the α-amylase from *Streptococcus bovis* on the cell surface. As the anchor protein, PgsA from *Bacillus subtilis* was fused to the N-terminus of the α-amylase. A lysine producer displaying this fusion protein on its cell surface produced 6.04 g/L of lysine with a conversion yield of 18.89% on starch; this titer and yield are higher than those obtained in glucose medium (Tateno et al. 2007). On the other hand, glycerol, the main by-product of biodiesel production, is a potential carbon source for biotechnological processes. *C. glutamicum* has been engineered to express the *E. coli* glycerol utilization genes *glpF*, *glpK*, and *glpD* so that it can grow on glycerol. This has allowed lysine production from glycerol with a yield of 19% (Rittmann et al. 2008).

Furthermore, *C. glutamicum* is being engineered to use lignocellulose as a feedstock. Although this organism is unable to utilize the pentoses xylose and arabinose, components of lignocellulose, a successful attempt has been made to implement the respective pathways that would allow growth on lignocellulose. A xylose-utilizing *C. glutamicum* strain has been constructed that expresses the *xylA* and *xylB* genes from *E. coli* on a high-copy plasmid (Kawaguchi et al. 2006). Similarly, heterologous expression of the *E. coli* arabinose-utilizing pathway in *C. glutamicum* resulted in a strain that is able to grow on arabinose (Kawaguchi et al. 2008). Recently, lysine production from rice straw hydrolysate has been demonstrated using a *C. glutamicum* strain which expresses the endogenous xylulose kinase gene from *C. glutamicum*

itself and the xylose isomerase gene from *Xanthomonas campestris*, together with the *E. coli araBAD* genes (Meiswinkel et al. 2013).

4

Genome Breeding of Lysine Producers

As has been described above, a long history of strain development for lysine production has resulted in a variety of industrially useful mutants (Table 5.1). Yet most of these industrial producers remain veiled in mystery because the causative mutations that enable them to be so useful are unknown. In search of a global understanding of the mechanisms that would permit lysine hyperproduction and more efficient lysine production, extensive research has recently been directed toward analyzing the genomes of the current producers, which has resulted in a novel methodology called "genome breeding" (Ohnishi et al. 2002; Ikeda and Nakagawa 2003; Ikeda et al. 2005, 2006; Lee et al. 2012; Kim et al. 2013; Wu et al. 2015). Genome breeding aims to reengineer more efficient producers using knowledge regarding the mutations that have accumulated over decades of industrial strain development. Specifically, this approach starts with decoding the genomes of classical industrial producers to identify the important genetic traits that distinguish them from their wild-type ancestors and progresses to systematically assembling the beneficial genetic properties in a single wild-type background (Fig. 5.5). With this strategy, it is possible to create a defined mutant that carries a minimal set of essential mutations for high-level production, while rationalizing production mechanisms at the same time. The procedure and impact of this approach are described here.

4.1

First Stage: Genome Analysis of a Classical Production Strain

C. glutamicum B-6 (Table 5.1) is a lysine-hyperproducing strain that originated from the wild-type strain ATCC 13032 but has since undergone decades of conventional mutagenesis and screening. As a useful producer, its genome is well worth studying. Whole-genome sequencing of this strain revealed that more than 1,000 mutations have accumulated in its genome. This means that between 50 and 100 mutations were introduced at every round of mutagenesis. The mutation types were mostly G·C→A·T transitions with a small fraction of A·T→G·C transitions (Ohnishi et al. 2008). About 60% of them were mutations causing amino acid substitutions. Metabolic pathway mapping of the mutations showed them to be widely distributed throughout the metabolic pathways including the central metabolism and the amino acid-biosynthetic pathways. Allele-specific PCR revealed the history of the mutations by identifying the step in which each mutation was introduced during the multiple rounds of mutagenesis. This form of analysis allowed us to associate genotypes with phenotypes, and thereby made it easy to select the positive mutations that have been beneficial for production.

4.2

Second Stage: Identification of Basal Mutation(s) Causing Lysine Production

The first step toward genome breeding was to identify the basal mutation(s) that conferred the ability to produce lysine on wild-type *C. glutamicum*. In general, such a basal mutation(s) is considered to exist in the relevant terminal pathways and at or near the earliest step in the phylogenetic tree of strain development. In the case of strain B-6, there were six point mutations identified, one in each of the *hom*, *lysC*, *dapE*, *dapF*, *thrB*, and *thrC* genes of the relevant

terminal pathways (Fig.5.6). The first two of these, that is, the *hom* mutation (V59A, designated *hom59*) and the *lysC* mutation (T311I, designated *lysC311*), existed at the earliest and second earliest steps in the phylogenetic tree, respectively. The presence of the *hom59* mutation and the *lysC311* mutation in the wild-type genome resulted in lysine accumulation at 10 and 55 g/L, respectively, in fed-batch fermentation using a glucose medium (Table 5.2), revealing that both mutations were basal mutations (Ohnishi et al. 2002). Comparative phenotypic analyses also clarified the relationship between the genotype of *hom59* and the phenotype of a partial requirement for homoserine, as well as that between the genotype of *lysC311* and the phenotype of resistance to AEC.

4.3

Third Stage: Selection for a Wild-type Background with Best Performance

C. glutamicum and its subspecies, known as glutamic acid-producing bacteria, have previously been classified as belonging to different genera or species, such as *Brevibacterium lactofermentum* and *Corynebacterium acetoacidophilum* (Liebl et al. 1991). Although they are currently classified as belonging to the original species *C. glutamicum* (Kinoshita 1999), these different wild-type strains exhibit considerable phenotypic diversity. Therefore, in the genome breeding process, it is important to start from the wild-type background with the best industrial performance because the intrinsic properties of the host strain chosen at the beginning of the process can have a significant impact on the ultimate outcome. For this purpose, the basal mutation *lysC311*was introduced into six representative *C. glutamicum* wild-type strains, including ATCC 13869 (formerly known as *B. lactofermentum*) and ATCC 13870 (formerly known as *C. acetoacidophilum*). The resulting isogenic mutants were then screened for their

abilities to produce lysine using jar fermenters under both traditional 30˚C conditions and suboptimal 40˚C conditions. The six *lysC* mutants all produced large amounts of lysine at both temperatures, but at different levels with respect to final titers and productivities (Ohnishi and Ikeda 2006). Through this evaluation, *C. glutamicum* ATCC 13032, a parental wild-type strain of strain B-6, was chosen as the best background with which to begin the process because its *lysC* mutant exhibited the highest titer and productivity under both temperatures among the six mutants.

4.4

Fourth Stage: Assembling Beneficial Mutations

The two basal mutations *hom59* and *lysC311* were assembled in the best background strain, ATCC 13032, which led to a synergistic effect on lysine production and the accumulation of 80 g/L of lysine (Table 5.2) (Ohnishi et al. 2002). No other mutations identified in the terminal pathways as coexisting with *hom59* and *lysC311* were effective for lysine production. Thus, the next task was to evaluate mutations positioned metabolically upstream, specifically in the central metabolism. In the genome of strain B-6, there were 14 point mutations identified in the genes relevant to central metabolism (Fig. 5.6). These mutations were separately evaluated for their effects on lysine production under the *hom59* and *lysC311* background. Eventually, three specific mutations, *pyc458* (P458S) in the anaplerotic pathway (Ohnishi et al. 2002), *gnd361* (S361F) in the pentose phosphate pathway (Ohnishi et al. 2005), and *mqo224* (W224opal) in the TCA cycle (Mitsuhashi et al. 2006), were identified as beneficial mutations for improved production (Fig. 5.6). Likewise, *leuC456* (G456D) in the leucine-biosynthetic pathway was defined as the beneficial mutation from among mutations in other metabolic pathways (Hayashi et al. 2006a).

These four beneficial mutations were then assembled one by one under the *hom59* and *lysC311* background, which resulted in step-wise increases in lysine production and a final titer of 100 g/L (Table 5.2) (Ikeda et al. 2006; Ikeda and Takeno 2013).

4.5

Performance of the Reengineered Strain

The reengineered strain, designated AGL-6 (Table 5.2), is, so to speak, a minimally mutated strain from which all undesirable mutations accumulated in the genome of strain B-6 were eliminated, and thus has several advantages over the classical producer. One of these is its high rate of growth and sugar consumption. The new strain can consume 250 g/L of glucose within 30 h, a rate comparable to that of the wild-type strain. This allows the fermentation period to be shortened to nearly half of that traditionally required (Ohnishi et al. 2002). A second advantage is improved stress-tolerance, because the reengineered strain is assumed to inherit the robustness of the wild-type strain. The new strain indeed exhibited efficient lysine production at a suboptimal temperature of 40˚C and achieved a titer of 100 g/L after only 30 h of jar fermenter cultivation, whereas strain B-6 could not work at all above 35˚C (Ohnishi et al. 2003; Ikeda et al. 2006). This performance allows for the reduction of cooling costs, and furthermore, makes cost-effective manufacture feasible in tropical regions with easy access to low-cost carbon sources. In the future, assembling positive mutations derived from different lines of classical producers in a single wild-type background is expected to lead to more impressive results, as demonstrated in genome breeding for arginine production (Ikeda et al. 2009).

4.6

Rationalizing Lysine Production Mechanism

The coexistence of the two basal mutations *hom59* and *lysC311* in the wild-type genome exerted a synergistic effect on lysine production. The possible explanation for this is that the *lysC311* mutation, which causes partial deregulation of aspartokinase, would achieve greater deregulation of the enzyme with the help of the *hom59* mutation, which causes threonine limitation (Ohnishi et al. 2002). This cooperation is considered to be the fundamental mechanism of lysine production in the reengineered strain as well as the classical producer B-6. Increased production by the *pyc458* mutation in the anaplerotic enzyme pyruvate carboxylase can be explained by improved catalytic activity, thereby increasing precursor supply for lysine biosynthesis (Ohnishi et al. 2002). The *gnd361* mutation in the 6-phosphogluconate dehydrogenase gene obviously contributes to increased supply of NADPH, as enzymatic and metabolic flux analyses have revealed that the mutation alleviated the allosteric regulation of the enzyme and caused an 8% increase in carbon flux through the pentose phosphate pathway (Ohnishi et al. 2005). The *mqo224* mutation in the malate:quinone oxidoreductase gene is a nonsense mutation changing TGG, a tryptophan codon, into TGA, a stop codon. The resulting loss of enzyme activity is useful for lysine production. Since transcriptome analysis revealed a coordinated down-regulation of the TCA cycle genes as a consequence of a deletion in the *mqo* gene, the mechanism for increased lysine production can be rationalized by decreased flux of the TCA cycle, resulting in redirection of oxaloacetate into lysine biosynthesis (Mitsuhashi et al. 2006). On the other hand, the *leuC456* mutation in the leucine-biosynthetic pathway has been found to cause leucine limitation and give rise to the global induction of the amino acid biosynthesis genes through a stringent-like regulatory mechanism. This upregulation likely contributes to increased lysine production mainly

through increased expression of the *lysC* gene encoding the key enzyme aspartokinase (Hayashi et al. 2006a).

5

Holistic Metabolic Design for Optimizing Lysine Production

Progress in *C. glutamicum* genomics and so-called post-genome technologies has opened up new avenues for the development of various global analysis techniques, which have led to the use of in-silico modeling and simulations in planning further strain engineering. For example, integration of the annotated genome, the available literature, and various "omic" data have resulted in the construction of a genome-scale model of the *C. glutamicum* metabolic network (Kjeldsen and Nielsen 2009). This metabolic model, comprised of 446 reactions and 411 metabolites, helps predict metabolic fluxes during lysine production and growth under various conditions at levels that strongly reflect experimental values. The ability to predict the metabolic state associated with maximum production yield has been used in the rational design of high lysine-producing strains of *C. glutamicum* (Krömer et al. 2004; Becker et al. 2005; Wendisch et al. 2006).

Such systems-level approaches, when combined with the ever-accumulating metabolic engineering and genome breeding data, as well as metabolic flux profiling and modeling technologies, have made it possible to predict a minimum set of genetic modifications that would lead to the theoretically best flux scenario for optimum lysine production (Becker et al. 2011). Through a series of these processes, now known as systems metabolic engineering, it has been demonstrated that twelve genetic modifications in a wild-type genome led to the lysine hyper-producer LYS-12 (Table 5.1, Fig. 5.7). This strain was shown to give a final titer of 120

g/L with a yield of 55% on glucose after 30 h of 5-liter jar fermenter cultivation at 30ºC. Among the twelve modifications shown in Figure 5.7, six are relevant to this strain's increased flux through the lysine-biosynthetic pathway. These include the introduction of the *lysC311* and *hom59* mutations, duplication of the *ddh* and *lysA* genes, and overexpression of the *lysC* and *dapB* genes under a strong promoter. Three of the other six modifications are the introduction of the *pyc458* mutation, overexpression of the *pyc* gene under a strong promoter, and deletion of the *pck* gene, all of which cause increased flux towards oxaloacetate through anaplerotic carboxylation. Two of the remaining three modifications are overexpression of the *fbp* gene and the *zwf-opcA-tkt-tal* operon under strong promoters, both of which cause increased flux through the pentose phosphate pathway for NADPH supply. The last one, replacement of the start codon ATG by the rare GTG in the *icd* gene, reduces flux through the TCA cycle and thereby increases the availability of oxaloacetate. It is worth noting that the three specific mutations *lysC311*, *hom59*, and *pyc458* carried by strain LYH-12 were originally identified from the genome of the classical industrial producer *C. glutamicum* B-6 during the genome breeding process (Table 5.2).

6

Conclusions and Outlook

The global lysine market has expanded to more than 2 million metric tons through the combined effects of the development of fermentation technology and increased lysine demand. This market growth is expected to continue, considering the increases in both nutritional values and applications of this amino acid (Eggeling and Bott 2015). Currently, lysine fermentation, including strain development technology, biochemical engineering, and downstream processing, is the most advanced example of a bio-based production process.

This chapter has provided an overview of lysine production processes and typical production strains and summarized the history of lysine production technology. To the best of our knowledge, the best lysine fermentation performance ever achieved is 170 g/L after 45 h (Eggeling and Sahm 1999). However, it should be noted that the record titer was attained by a classical strain. This suggests that there is still a significant gap in performance between the best classical strain and strains metabolically engineered from wild type. It has not yet been possible to reproduce the record titer from scratch through rational approaches only, probably due to the existence of unknown mechanisms for hyperproduction. This means that there is a great deal more to learn from the genomes of classical strains. As we enter the genomic era, new possibilities emerge, including analysis of producer's genomes, leading to genome breeding, and systems metabolic engineering, leading to tailor-made cell factories with designed properties (Becker and Wittmann 2015). The next-generation strains are expected to be created through the synergy of these approaches, and through integrating the knowledge accumulated over decades of industrial strain development with the emerging technology of in-silico modeling and simulation.

At the same time, the lysine industry is beginning to consider sustainable and environmentally-friendly manufacturing systems in response to the continuing crisis of global warming. From this standpoint, the industry is expected to develop strains enabling the use of feedstocks that are renewable and that do not compete with human food or energy sources. The development of innovative technologies enabling reduction in effluents and wastes generated during fermentation and purification processes is also expected. Tackling these challenges will lead to a new era for the lysine industry.

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Table 5.1 Typical lysine-producing strains

Strain	Remarks	Culture conditions	Titer (g/L)	Yield (%)	Productivity (g/L/h)	Reference
Micrococcus glutamicus (*C. glutamicum*) No. 901	Hse^-	glucose 10% 28°C, 3-4 days	12.9	12.9	–	Nakayama et al. 1961
Brevibacterium flavum FA-3-115	AEC^r	glucose 10% 30°C, 72 h	31.8	31.8	–	Sano and Shiio 1970
B. flavum No. 22	AEC^r, CS^D, PK^D, HD^D, and FP^s	glucose 10% 30°C	51	51	–	Ozaki and Shiio 1983
C. glutamicum B-6	AEC^r, Rif ^{r}, SM^r, and AU^r	molasses 32°C, 48 h	100	–	2.1	Hirao et al. 1989
C. glutamicum H-8241	AEC^r, Rif ^{r}, SM^r, AU^r, NQ^r, $Iturin^r$, and TIT^r	sucrose 10% 32°C, 72 h	48	48	–	Nakano et al. 1994
C. glutamicum	Hse^-, Leu^-, and AEC^r	glucose 18% 27°C, 70 h	60	33.3	–	Sassi et al. 1996
C. glutamicum MH20-22B /pJC23	AEC^r (AK^{IS}) and Leu^- *dapA* on plasmid	glucose 10%	50	50	–	Eggeling et al. 1998
Brevibacterium lactofermentum AJ12592	Hse^-, AEC^r, CCL^r, and DOL^r	glucose 3.6% 31.5°C, 48 h	11.8	32.8	–	Yokomori et al. 1994
B. lactofermentum AJ12937	AEC^r, and Ala^-	glucose 31.5°C, 58 h	120.5	–	–	Shiratsuchi et al. 1995
B. lactofermentum AJ11082/pSSM30BS	AEC^r, Ala^-, and CCL^r sucrase gene on plasmid	sucrose 31.5°C, 35 h	95	–	–	Sugimoto et al. 1996
B. lactofermentum AJ11082/pCL and pPwm	AEC^r, Ala^-, and CCL^r *lysC*, *lysA*, and *ppc* on plasmids	glucose 10% 31.5°C, 72 h	45.2	45.2	–	Hayakawa et al. 1998
B. lactofermentum AJ11082/pCABL and pORF1	AEC^r, Ala^-, and CCL^r *lysC*, *dapA*, *dapB*, *lysA*, and *aspC* on plasmids	glucose 10% 31.5°C, 72 h	48.8	48.8	–	Araki et al 1999
B. lactofermentum AJ3990/pHSG::THYB	transhydrogenase gene on plasmid	glucose 3.6% 31.5°C, 72 h	14.5	40.3	–	Kojima and Totsuka 2002
C. glutamicum AGL-6	Reengineered strain carrying 6 mutations on genome (*hom59*, *lysC311*, *pyc458*, *gnd361*, *mqo224*, and *leuC456*)	glucose 40°C, 30 h	100	40	3.3	Ikeda and Takeno 2013

C. glutamicum LYS-12	Defined strain with 12 genetic modifications on genome (*hom59*, *lysC311*, *pyc458*, duplication of *ddh* and *lysA*, overexpression of *lysC*, *dapB*, *pyc*, *fbp*, and *zwf-opcA-tkt-tal*, deletion of *pck*, and attenuation of *icd*)	glucose 30°C, 30 h	120	55	4.0	Becker et al. 2011
C. glutamicum	classical producer	glucose 45 h	170	–	3.8	Eggeling and Sahm 1999
C. glutamicum Lys5-8	*lysC*^{C932T}, *pyc*^{G1A,C1372T}, *hom*^{T176C}, Δ*ilvN*$_{C-T}$, Δ*aceE::lysA*, Δ*alaT::dapA*, Δ*avtA::ddh*, Δ*ldhA::dapB*, Δ*mdh::asd*, Δ*gapA::gapC*, Δ*pck::lysC* ^{C932T}	glucose 48 h	130.82	47.06	2.73	Xu et al. 2014
Corynebacterium thermoaminogenes AJ12521	thermotolerant *Corynebacterium* AEC^r	glucose 10% 43°C, 72 h	30	30	–	Murakami et al. 1993
E. coli W3110*tyrA* /pCABD2	*dapA*, *lysC*, *dapB*, and *ddh* on plasmid	glucose 4% 37°C, 30 h	12.23	30.6	–	Kojima et al. 1995
E. coli AJ13069 /pSYBJEl	AEC^r *ybjE* on plasmid	glucose 4% 37°C, 27 h	7.6	19	–	Ueda et al. 2005
E. coli NT1003	Thr$^-$and Met$^-$ *ppc*, *pntB*, and *aspA* on plasmid	glucose 72h	134.9	45.4	1.9	Ying et al. 2014
Bacillus methanolicus NOA2#13A52-8A66	thermotolerant methylotroph Hse$^-$, AEC^r, and Lys^r	methanol 50°C, 60 h	47	–	–	Lee et al. 1996

Hse, L-homoserine; Leu, L-leucine; Ala, L-alanine; Lys, L-lysine; Thr, L-threonine; Met, DL-methionine; AEC, *S*-(2-aminoethyl)-L-cysteine; FP, β-fluoropyruvate; Rif, rifampicin; SM, streptomycin; AU, 6-azauracil; NQ, naphtoquinoline; TIT, 3, 3', 5-L-triiodo-L-thyronine; CCL, α-chlorocaprolactam; DOL, $N^{\alpha}N^{\varepsilon}$-dioctanoyl-L-lysine; AK, aspartokinase; CS, citrate synthase; PK, pyruvate kinase; HD, homoserine dehydrogenase; DDH, *meso*-diaminopimelate

dehydrogenase; ^{r}, resistant; ^{s}, sensitive; ^{D}, decreased or deficient activity; ^{IS}, insensitive to feedback regulation; $^{-}$, auxotroph

Table 5.2 Phylogeny of defined lysine producers reengineered by the genome breeding approach

Strain	Genotype	Titer (g/L)	Time (h)	Productivity (g/L/h)
ATCC 13032 (Wild-type)	_	0	30	0
HD-1	*hom59*	10	30	0.33
AK-1	*lysC311*	55	30	1.83
AHD-2	*hom59, lysC311*	80	30	2.67
AHP-3	*hom59, lysC311, pyc458*	85	30	2.83
APG-4	*hom59, lysC311, pyc458, gnd361*	90	30	3.0
AGM-5	*hom59, lysC311, pyc458, gnd361, mqo224*	94	30	3.13
AGL-6	*hom59, lysC311, pyc458, gnd361, mqo224, leuc456*	100	30	3.33

Cultivations for lysine production were carried out at 40°C in 5-L jar fermenters 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 glucose, NH_4Cl, and D-biotin was continuously fed until the total amount of sugar in the medium reached 25% (Ohnishi et al. 2002; Ikeda et al. 2006)

Fig. 5.1 Changes in estimated global markets (*bars*) and prices (*circles*) for lysine during the past decade

Fig. 5.2 Targets of metabolic engineering for lysine production

Fig. 5.3 Design of *S. mutans*-type redox metabolism in *C. glutamicum*. Endogenous NAD-dependent glyceraldehyde 3-phosphate dehydrogenase (*GapA*) of *C. glutamicum* was replaced with nonphosphorylating NADP-dependent glyceraldehyde 3-phosphate dehydrogenase (*GapN*) of *S. mutans*, leading to a *C. glutamicum* strain with an NADPH-generating glycolytic pathway

Fig. 5.4 Possible mechanism for an increase in lysine production, activated by switching the glucose transport systems from the PTS to the *iolT1*-specified non-PTS route. Considering that lysine production is generally limited by the pyruvate carboxylase (*Pyc*) reaction (Peters-Wendisch et al. 2001), it is likely that the increased availability of phosphoenolpyruvate (*PEP*) relative to pyruvate (*Pyr*) in the non-PTS strain contributes to a better balanced supply of carbon from the central metabolism into the lysine-biosynthetic pathway through the two anaplerotic reactions involving pyruvate carboxylase and PEP carboxylase (*Ppc*)

Fig. 5.5 Reengineering of defined lysine producers by genome breeding. This approach starts with comparative genomic analysis to identify mutations and eventually leads to creation of a defined mutant that carries a minimal set of beneficial mutations (*e.g., hom59, lysC311*) and no undesirable mutations (x)

Fig. 5.6 Mutated genes identified in the lysine-biosynthetic pathways and central carbon metabolism through whole-genome sequencing of *C. glutamicum* B-6. Both *grey* and *black boxes* represent genes in which mutations were identified. The *five specific mutations* indicated above the *black boxes* were defined as positive mutations for lysine production. Abbreviations and symbols for genes are as described in the previous report (Hayashi et al. 2002)

Fig. 5.7 Systems metabolic engineering for lysine production. On the basis of the metabolic blueprint, twelve genetic modifications were introduced into a wild-type genome, which resulted in the lysine hyperproducer *C. glutamicum* LYS-12. Abbreviations and symbols for genes are as described in the previous report (Hayashi et al. 2002)

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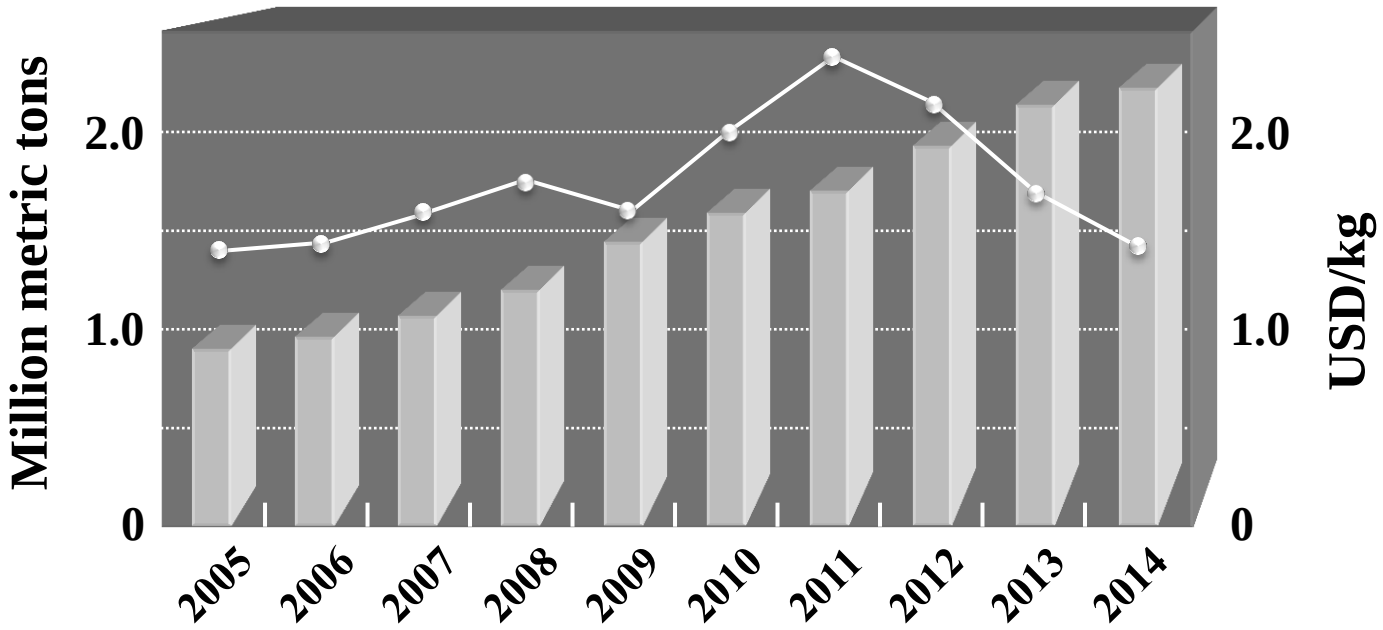
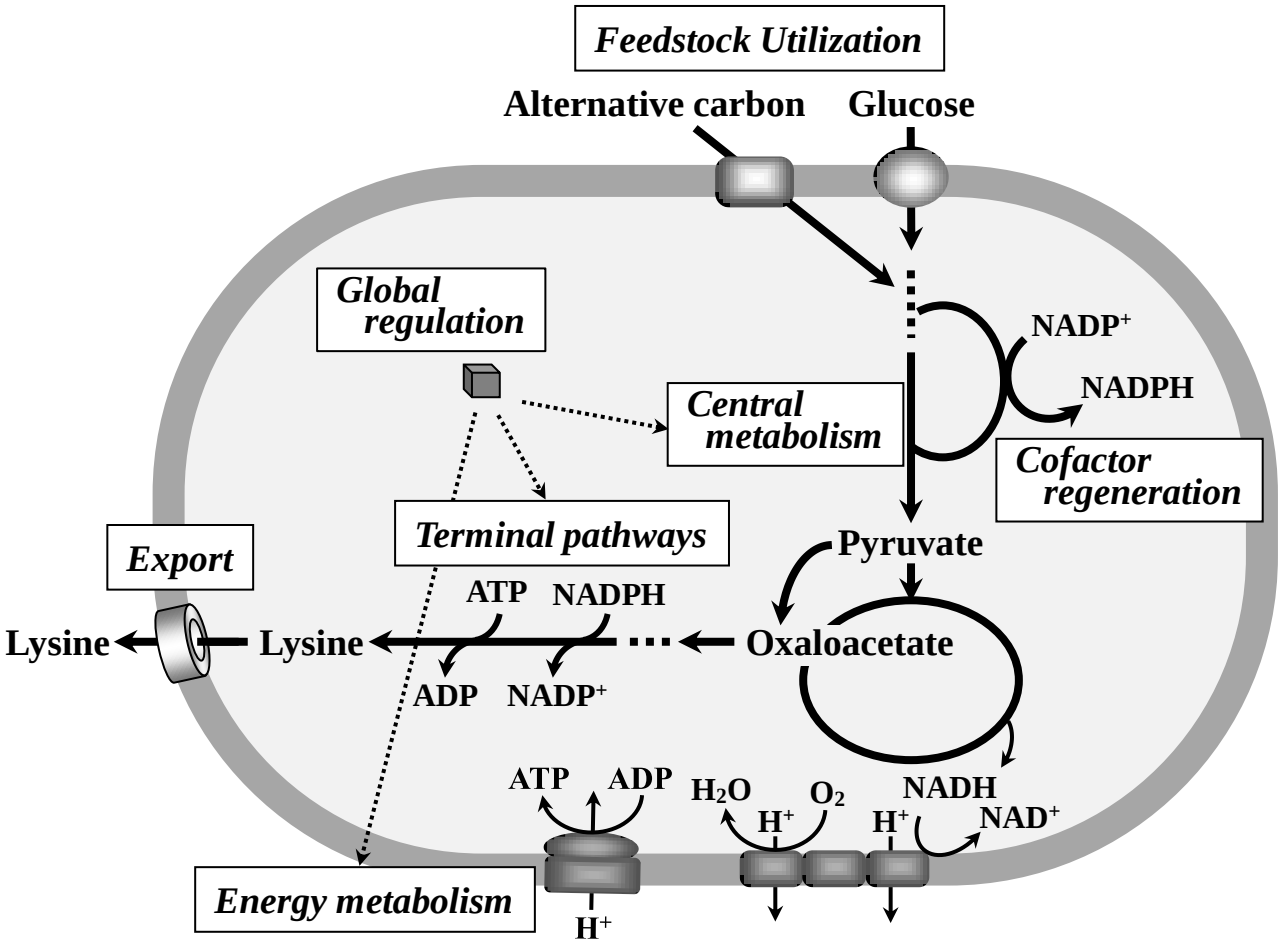

Million metric tons
2.0
1.0
0
2.0
1.0
0
USD/kg
2005
2006
2007
2008
2009
2010
2011
2012
2013
2014

Fig. 5.1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Alternative carbon
Glucose
Global regulation
NADP+
NADPH
Central metabolism
Cofactor regeneration
Terminal pathways
Pyruvate
Export
ATP
NADPH
Lysine
Lysine
Oxaloacetate
ADP
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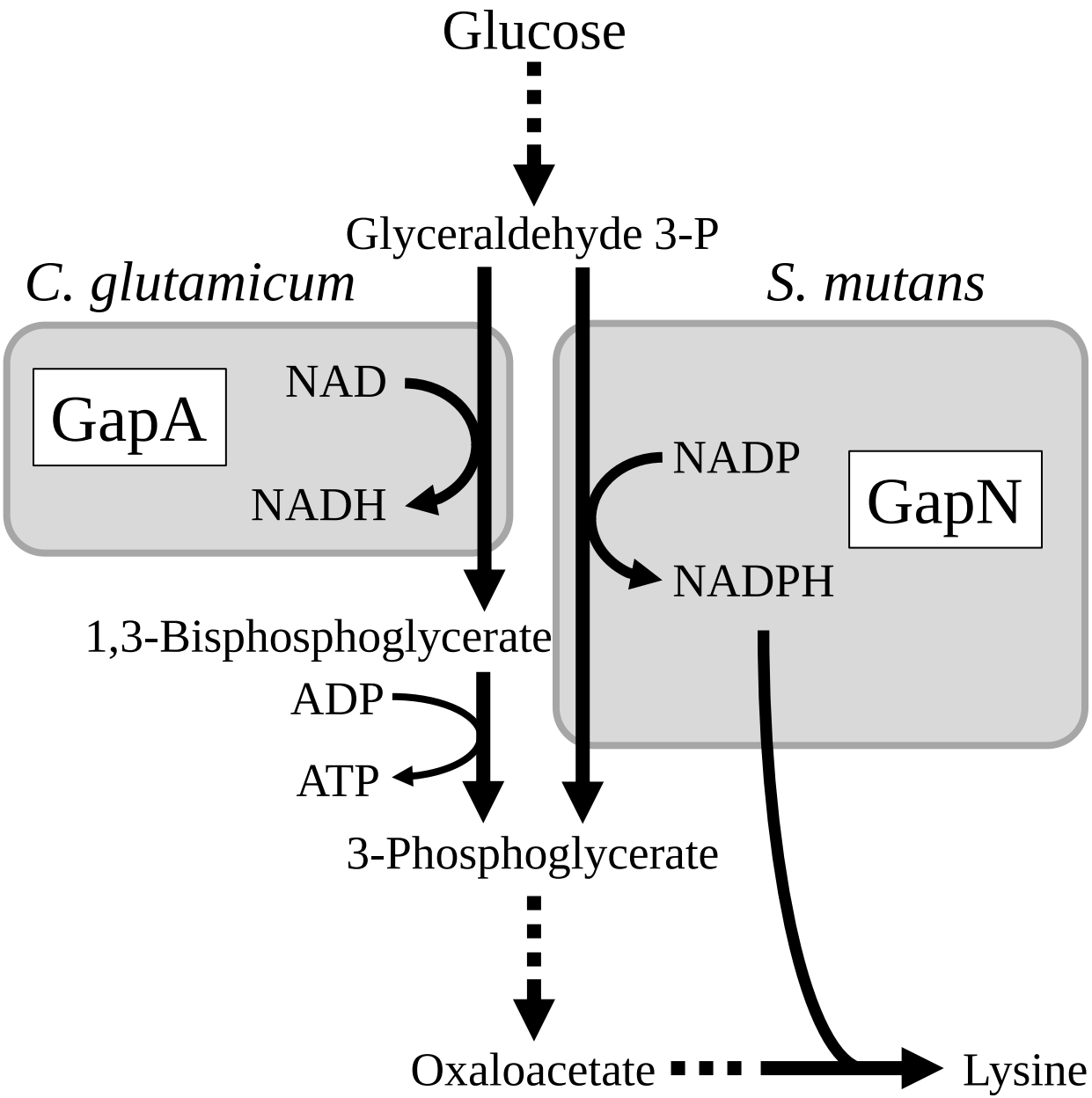
ATP
ADP
H2O
O2
NADH
H+
H+
NAD+
Energy metabolism
H+

Fig. 5.2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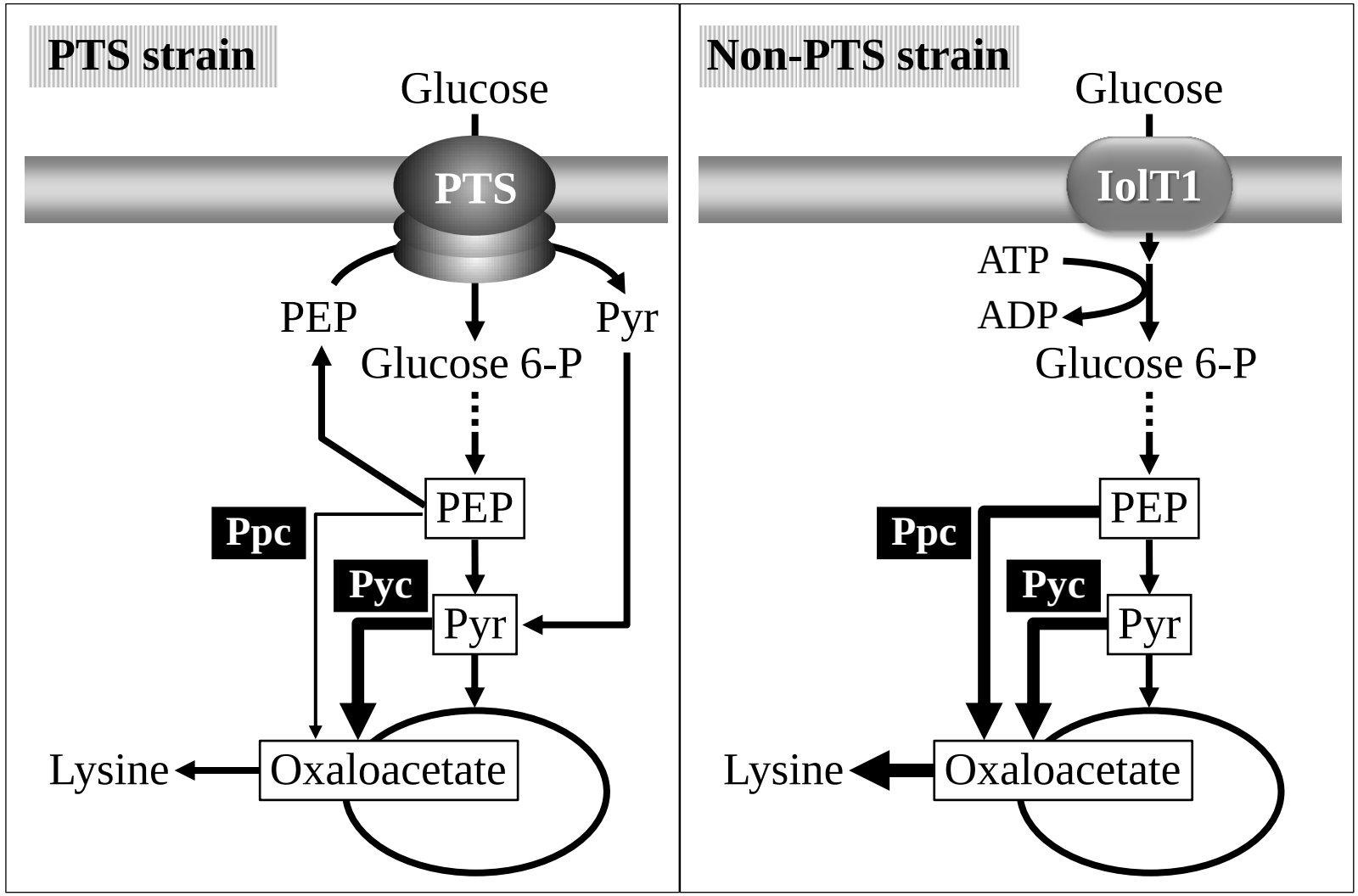
Glyceraldehyde 3-P
C. glutamicum
S. mutans
GapA
NAD
NADH
NADP
NADPH
GapN
1,3-Bisphosphoglycerate
ADP
ATP
3-Phosphoglycerate
Oxaloacetate
Lysine

Fig. 5.3


PTS strain
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PTS
PEP
Pyr
Glucose 6-P
PEP
Ppc
Pyc
Pyr
Lysine
Oxaloacetate
Non-PTS strain
Glucose
IolT1
ATP
ADP
Glucose 6-P
PEP
Ppc
Pyc
Pyr
Lysine
Oxaloacetate

Fig. 5.4

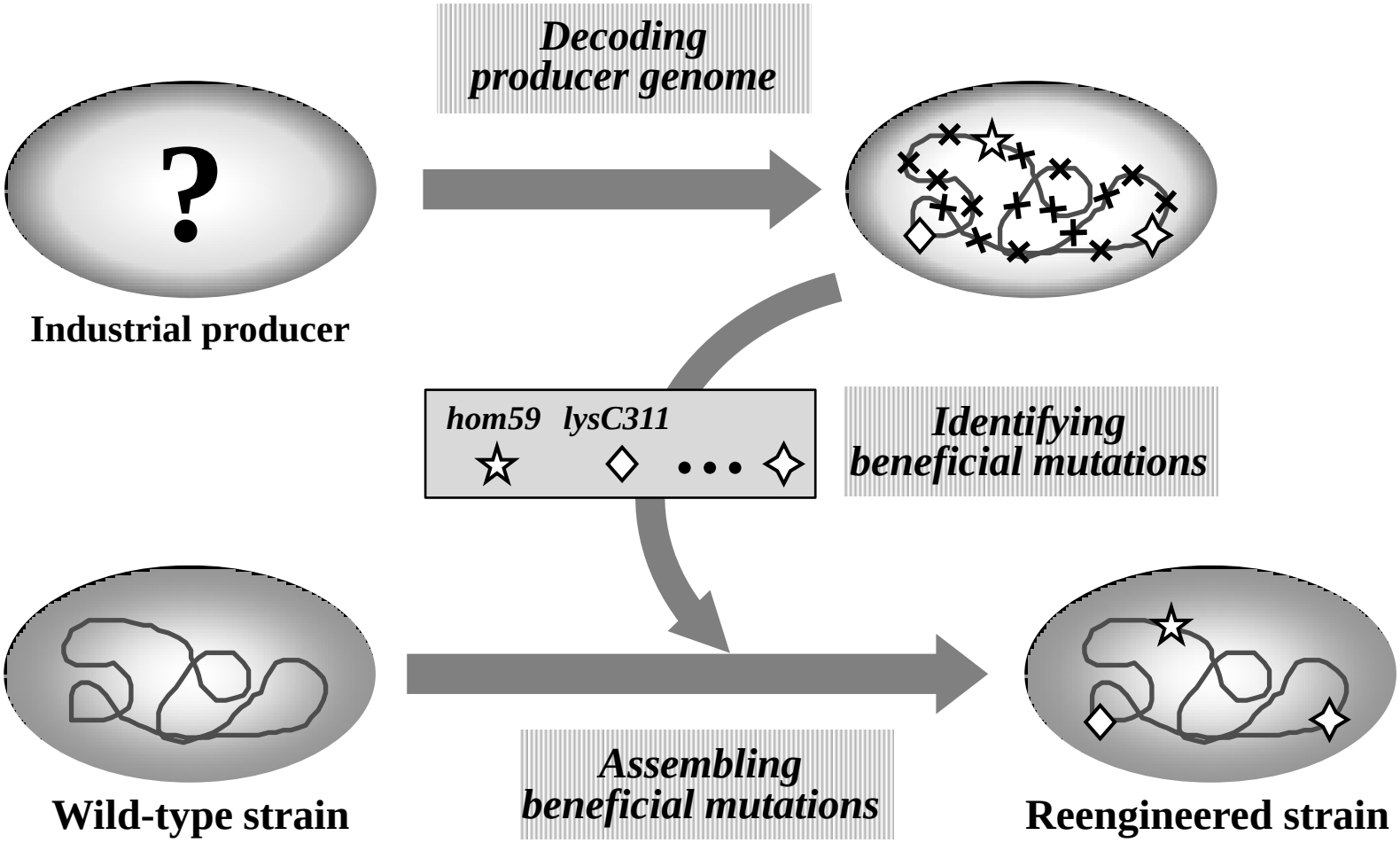
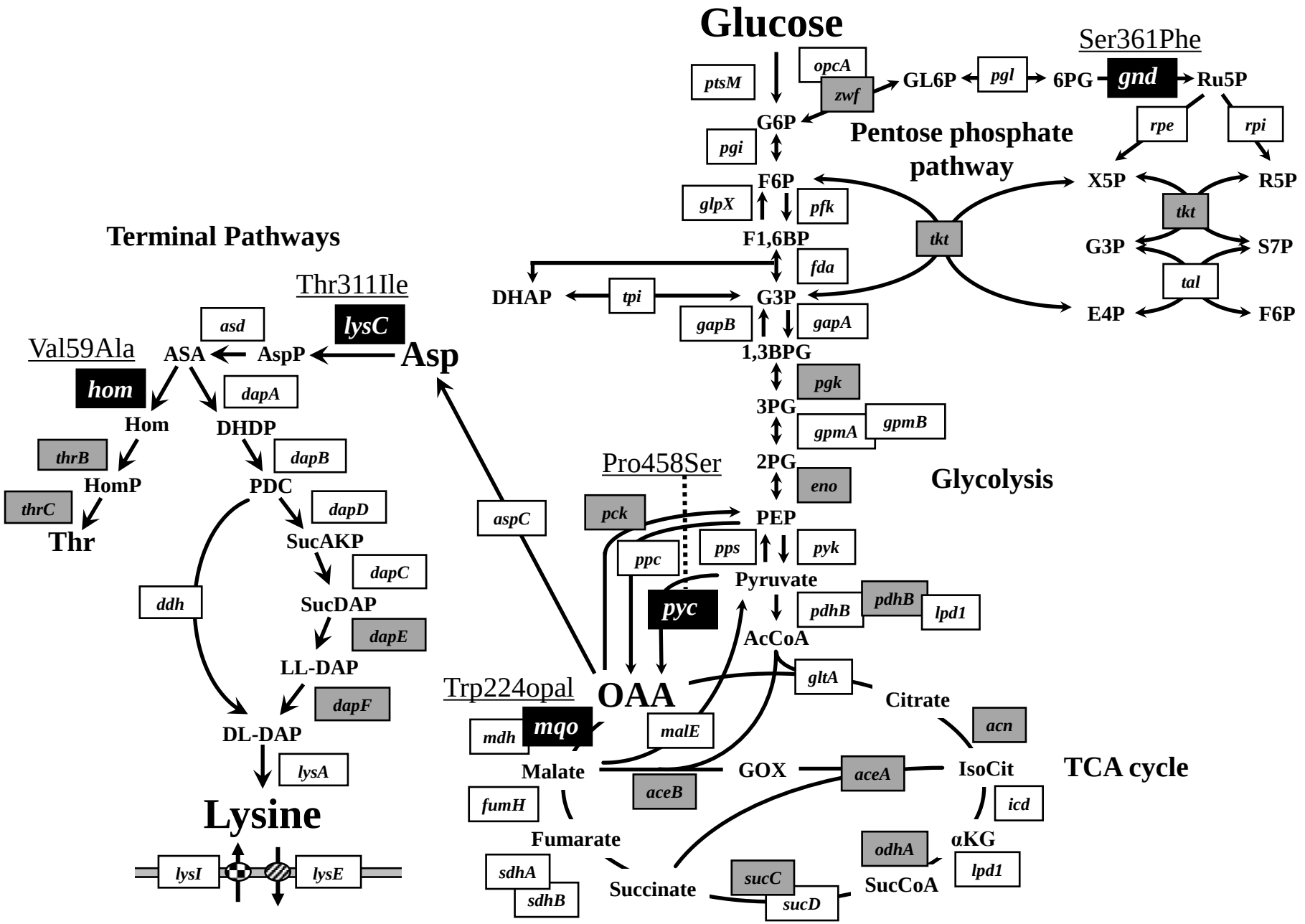

Decoding
producer genome
?
Industrial producer
hom59 lysC311
Identifying
beneficial mutations
Wild-type strain
Assembling
beneficial mutations
Reengineered strain

Fig. 5.5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zwf
GL6P
pgl
6PG
gnd
Ru5P
G6P
rpe
rpi
Pentose phosphate pathway
pgi
F6P
X5P
R5P
glpX
pfk
tkt
F1,6BP
G3P
S7P
fda
tal
DHAP
tpi
G3P
E4P
F6P
gapB
gapA
1,3BPG
Terminal Pathways
Thr311Ile
lysC
asd
Val59Ala
ASA
AspP
Asp
pgk
hom
dapA
3PG
Hom
DHDP
gpmA
gpmB
thrB
dapB
Pro458Ser
2PG
HomP
PDC
eno
Glycolysis
thrC
dapD
aspC
pck
PEP
Thr
SucAKP
pps
pyk
ppc
dapC
Pyruvate
ddh
SucDAP
pyc
pdhB
pdhB
lpd1
dapE
AcCoA
LL-DAP
gltA
Trp224opal
OAA
Citrate
dapF
mqo
acn
DL-DAP
mdh
malE
Malate
GOX
aceA
IsoCit
TCA cycle
lysA
aceB
fumH
icd
Lysine
Fumarate
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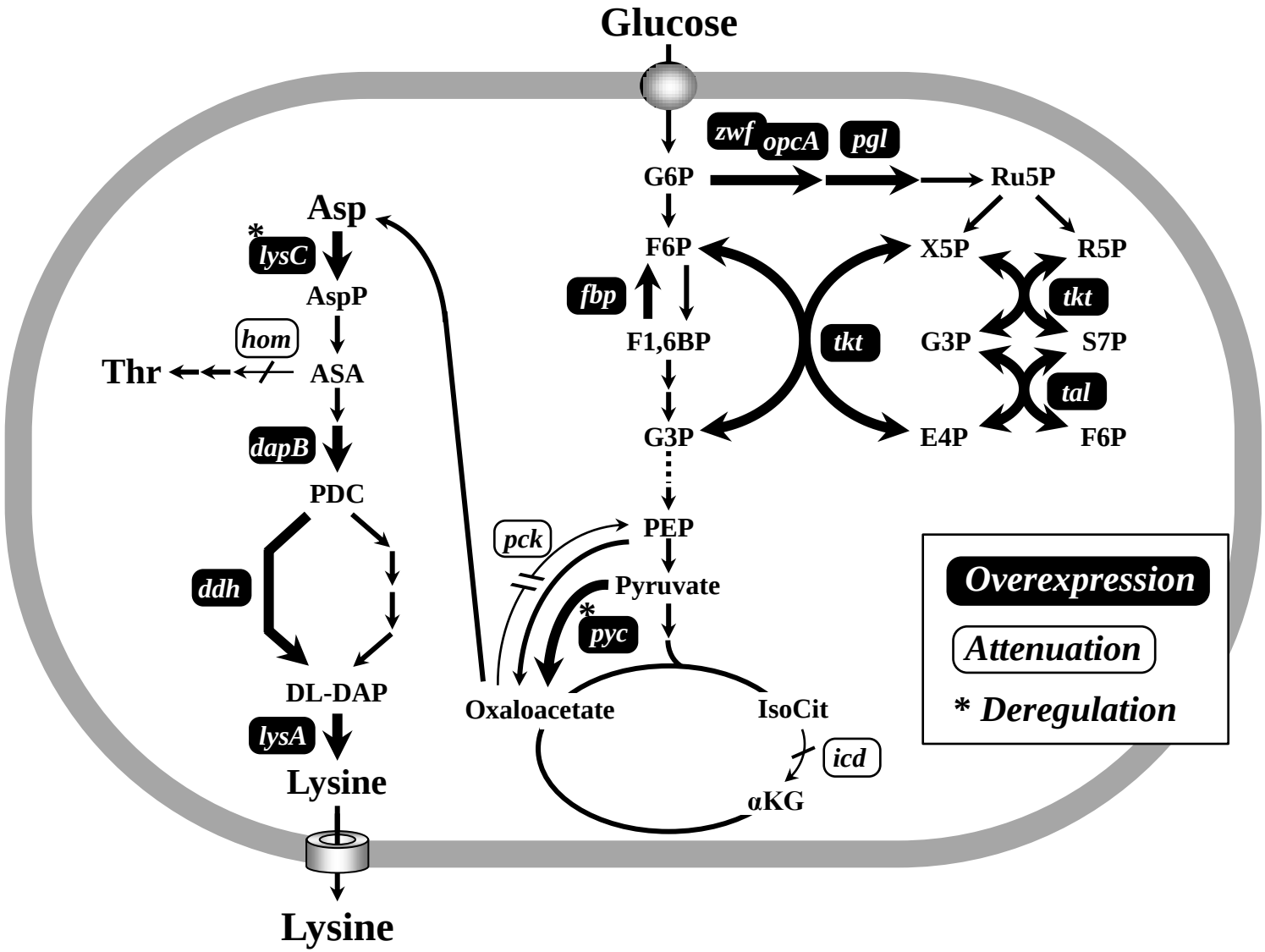
lysE
sdhA
lpd1
Succinate
sucC
SucCoA
sdhB
sucD

Fig. 5.6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pgl
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Asp
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lysC
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R5P
AspP
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hom
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tkt
G3P
S7P
Thr
ASA
tal
dapB
G3P
E4P
F6P
PDC
pck
PEP
ddh
Pyruvate
*
pyc
DL-DAP
Oxaloacetate
IsoCit
lysA
icd
Lysine
αKG
Lysine
Overexpression
Attenuation
* Deregulation

Fig. 5.7