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Amino Acid Fermentation

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Amino Acid Fermentation

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Preface

Amino acid fermentation is a field of applied microbiology that started approximately 50 years ago and it has a unique history in research and industrial application. In this field, both research and application progressed together. The former includes the elucidation of biosynthetic pathways and its regulation mechanism, and the latter, the optimization of the strain, fermentation conditions, and isolation process of the produced amino acid.

In 1908, a Japanese professor, Kikunae Ikeda, discovered glutamate as an umami substance, and a new seasoning, monosodium glutamate (MSG), was commercialized. In the early days of its manufacture, glutamate was extracted from the hydrolysate of wheat or soybean. However, with the increasing demand for MSG throughout the world, a more efficient production system was required.

Amino acid fermentation emerged as one of the candidates for a new production method of glutamate. Several methodologies for the new production system, including chemical synthesis and enzymatic conversion, were invented. The fermentation method was among the choices, and a producer strain was sought. However, in those days, many researchers felt that such a strain did not exist. The fermentation products known then were ethanol, lactate, acetone-butanol, and similar substances. They are the fermentation end products formed under anaerobic conditions. In contrast, glutamate is one of the building blocks of proteins. Therefore, it was not expected to find bacteria that would overproduce and excrete such an important compound. Therefore, it was truly an epoch-making success to find a glutamate-producing bacterium, *Corynebacterium glutamicum* (formerly *Micrococcus glutamicus*). With this discovery, today all the commercially supplied glutamate is produced by the fermentation method.

Subsequently, the target product has been extended from glutamate to other amino acids. Apart from being used as seasoning agents, these amino acids are of great value for medical use and nutrition in feed, as well as for cosmetics and other chemical and biological functions. For breeding the producer of each amino acid, "the conventional breeding method" was used, in which auxotrophic or analog-resistant mutants were derived to screen the desired producer. Later, the application of

genetic engineering techniques extended the host bacteria used for the production of substances to other than *C. glutamicum*, including *Escherichia coli*. The increased capacity to supply these amino acids at reasonable prices created new markets. For example, lysine and other amino acids such as threonine, tryptophan, and valine are used as feed additives by livestock farmers. At present, lysine is produced in larger volume than glutamate. As a consequence, a new field of research and industry, so-called amino acid fermentation, has been established.

In this book, 50 years of history, the present situation, and future prospects for amino acid fermentation as well as recent advances in fermentation research of several amino acids are described.

Atsushi Yokota
Masato Ikeda

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Part I
Situation and History of Amino Acid Fermentation

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Present Global Situation of Amino Acids in Industry

Naoto Tonouchi and Hisao Ito

Abstract At present, amino acids are widely produced and utilized industrially. Initially, monosodium glutamate (MSG) was produced by extraction from a gluten hydrolysate. The amino acid industry started using the residual of the lysate. The discovery of the functions of amino acids has led to the expansion of their field of use. In addition to seasoning and other food use, amino acids are used in many fields such as animal nutrients, pharmaceuticals, and cosmetics. On the other hand, the invention of the glutamate fermentation process, followed by the development of fermentation methods for many other amino acids, is no less important. The supply of these amino acids at a low price is very essential for their industrial use. Most amino acids are now produced by fermentation. The consumption of many amino acids such as MSG or feed-use amino acids is still rapidly increasing.

Keywords Animal nutrients, Cosmetics, Function of amino acids, Gluten hydrolysate, Industrial use, Pharmaceuticals

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1 Introduction

Amino acids are the building blocks of proteins. Each amino acid molecule has an amino group and a carboxyl group. At present, amino acids are widely produced and used in many fields. Table 1 shows the production methods and industrial applications of each amino acid. There are many production methods such as extraction, chemical synthesis, fermentation, and enzymatic conversion. Fermentation is suitable for the large-scale production of optically active compounds. Most amino acids are now produced by fermentation, although some such as glycine or DL-methionine are still produced by chemical synthesis or enzymatic production. On the other hand, the field of amino acids is not limited to seasoning. In the livestock industry, pharmaceuticals, and cosmetics, various amino acids are also used in large amounts. In this chapter, we review the present situation of the amino acid industry in some fields along with their functions.

2 Amino Acids and Fermentation

Amino acids are the building blocks of proteins. The amino group of an amino acid molecule can connect to the carboxyl group of another amino acid. The chained amino acids are called proteins. Our bodies contain hundreds of thousands of proteins (Fig. 1). Proteins are the building blocks of our body: skin, hair, muscles, bones, internal organs, and even red and white blood cells are made up of proteins. They also perform other inevitable functions such as providing nutrition, protecting the body (through the immune system), and controlling metabolic reactions. Proteins make up approximately 20% of the human body. There are more than 100,000 types of proteins; however, they are all made up of only 20 types of amino acids. It can therefore be said that amino acids are the essence of life. Previously, in Japan, the term "amino acid" was only understood as a nutrient. In 1995, the term was recognized by only less than 5% of the population. However, at the beginning of the twenty-first century, amino acids were labeled as "good for health," and more than 90% of the population recognized the term by 2008 [1]. In the nutrition field, 20 amino acids are divided into two categories: essential (indispensable) amino acids and nonessential (dispensable) amino acids. Essential amino acids are not produced by our body and must be obtained through food. It is often assumed that only essential amino acids are important, whereas nonessential amino acids are not so important. However, this assumption has recently been proven to be untrue.

Table 1 Production methods and industrial applications of amino acids

	Production method				Industrial application				
Amino acid	Fermentation	Enzymatic conversion	Chemical synthesis	Extraction	Pharmaceuticals	Food	Cosmetics	Animal feed	Other industrial uses
L-Alanine		●	●		●	●	●		
DL-Alanine			●		●	●			●
L-Arginine	●				●	●	●	●	
L-Asparagine		●			●	●			
L-Aspartate		●			●	●	●		●
L-Citrulline	●		●	●	●	●	●		
L-Cysteine	●	●		●	●	●	●		
L-Glutamate (monosodium)	●				●	●		●	●
L-Glutamine	●				●	●		●	
Glycine			●		●	●	●	●	●
L-Histidine	●				●				
L-Isoleucine	●				●	●			
L-Leucine	●			●	●	●			
L-Lysine (chloride)	●				●	●	●	●	●
L-Methionine		●	●		●	●	●		
DL-Methionine			●		●	●	●	●	
L-Ornithine	●		●		●	●			
L-Phenylalanine	●		●		●	●			●
L-Proline	●			●	●	●	●		
L-Serine	●	●		●	●		●		
L-Threonine	●				●	●	●	●	
L-Tryptophan	●	●			●	●		●	
L-Tyrosine				●	●				
L-Valine	●				●	●		●	

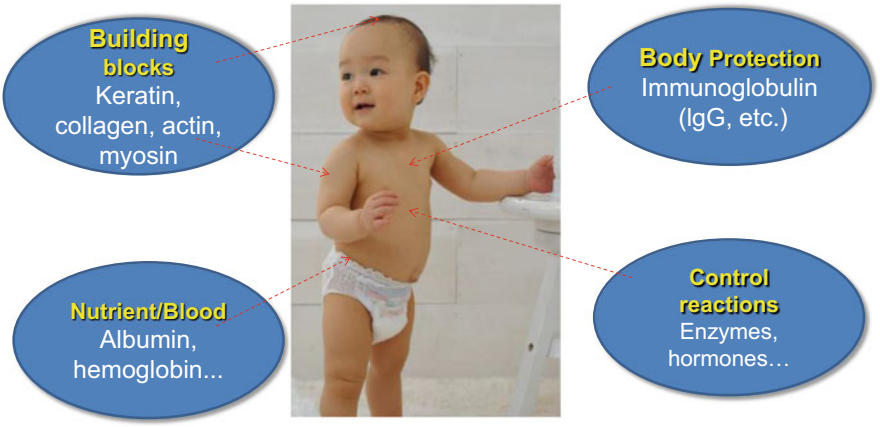

Fig. 1 Proteins in human body

From the physiological point of view, obtaining amino acids from food saves energy. Nonessential amino acids have many biological functions, not only as building blocks but also as intermediates in the metabolic system of the body (energy sources, nitrogen donors, and precursors of other compounds); therefore, we should be aware of the mechanism for synthesizing these amino acids.

Fermentation is a conversion process by microorganisms. There are many fermented foods worldwide, such as "miso" (bean paste) and soy sauce made from soybeans, paste and sauce made from fish or shrimp, "nata" (gelatinous pellicle) from coconut juice (nata de coco), yogurt and cheese from milk, "sauerkraut" (sour cabbage), and many kinds of pickles and alcohols made from local materials. Another type of fermentation is the conversion of sugars into useful compounds. Such fermentation results in the efficient production of the desired substances. It is effective in the production of optically active substances. Low-price carbohydrates such as starch, crude sugar, and molasses are used as the raw material.

3 Outline of the History of the Amino Acid Industry

In 1908, Prof. Kikunae Ikeda discovered glutamate as the umami substance. Following this, monosodium glutamate (MSG) was sold as seasoning in 1909. MSG was the first industrially commercialized amino acid. At that time, glutamate was extracted from the hydrolysate of wheat protein. In wheat, glutamic acid constitutes 30% of the total amino acids. Therefore, 70% is other amino acids. The applications of the remaining amino acids marked the start point of the amino acid industry. We started with a modest beginning from glutamic acid to more efficient use of that acid and expanded into other amino acids as well.

In 1935, the production of the first pharmaceutical product made with amino acids, "Histamine B," was started. Following this, in the 1950s, a highly purified

pharmaceutical-use amino acid product for infusions was launched. In the 1960s, feed-use amino acid production was started using the fermentation method. The fermentation process made it possible to produce large amounts of amino acids at a low cost. One of the important properties of these molecules is their reactivity. Amino acids express novel functions by reacting with other substances. For example, amino acid such as glutamate can react with fatty acids. The product, N-acyl glutamate, has a mild detergent activity and is used in the cosmetics field as a nonirritating soap. In recent years, many fundamental food and biotechnology businesses are being developed on the basis of the newly discovered physiological functions of amino acids.

4 Umami, Glutamate, and MSG

Umami is one of the five basic tastes (Table 2). It was discovered by Prof. Ikeda in 1908. A hundred years ago, only four basic tastes were recognized (sweet, sour, salty, and bitter). However, Prof. Ikeda thought that there was a fifth taste for foods. He found that glutamate is the umami substance. However, it was recognized worldwide only in the twenty-first century. One can easily imagine the other tastes: sweet is for the taste of sugar, sour is for acetate or citrate, salty is for sodium chloride, and bitter for caffeine or catechin. *But umami is not*. It is expressed as some word such as 'meaty', 'savory', 'brothy' or 'mouthfulness'. The mechanism of basic tastes is illustrated in Fig. 2. At the bottom of the tongue, there are some dots in a line; these are the nipples or papillae. At the bottom of each nipple, there are many buds, known as taste buds. On the surface of the taste buds, receptors for the basic tastes exist [2]. When umami substance binds to the taste buds, we perceive the taste. How do we perceive the taste? A signal is generated and transferred to the brain through the taste nerve. For tastes other than the basic ones such as hot, spicy, or astringent, the signal is different. It is transferred to the brain through another nerve system (the trigeminal nerve).

Umami is the signal of protein; it is the taste of digested protein. Glutamate is the most abundant amino acid and has the strongest umami taste. Similarly, sweet taste is the signal of carbohydrates (starch or other polysaccharides); the sweet taste of sugar comes from digested polysaccharides. Actually, the taste sense triggers the cephalic phase response to prepare the food digestion in the gut [3]. Characteristic of glutamate absorption at the intestine is very unique. Uptake of dietary free

Table 2 The five basic tastes

Taste	Examples of taste material	Implication of the taste
Sweet	Sugar (sucrose, glucose)	Carbohydrates (energy)
Sour	Acetate, citrate	Possibly unripe or rotten (caution)
Salty	Salt (sodium chloride)	Minerals
Bitter	Caffeine, catechin	Possibly toxic (warning)
Umami	Glutamate, nucleotide (inosinate, guanylate)	Proteins (body building)

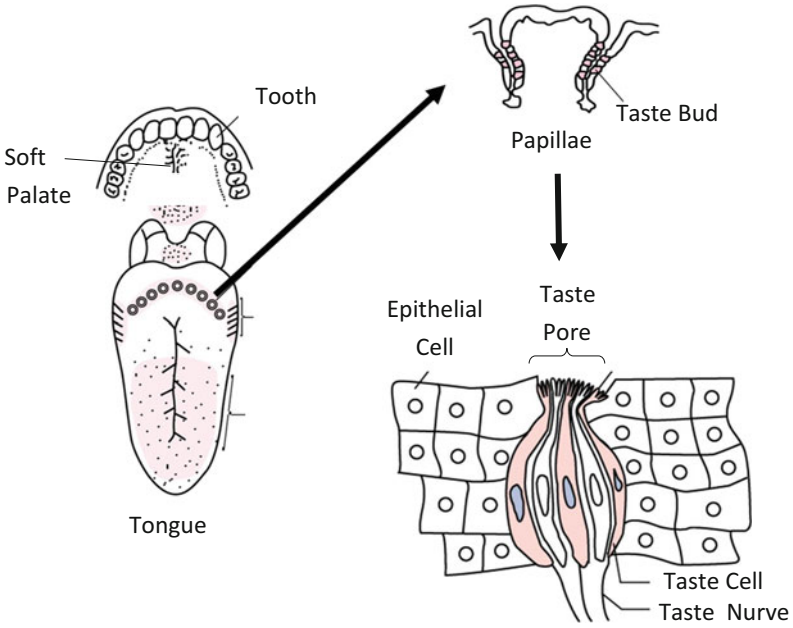

Fig. 2 Mechanism of basic taste perception

glutamate into the portal vein is less than 5%, and most of luminal is metabolized as an energy source within the intestinal epithelial cells [4].

In earlier days, most people in the Western countries did not understand the umami taste. They did not have any concept or equivalent word for umami. At first, in the USA, MSG was not considered a seasoning but a flavor enhancer. They described the taste as savory, meaty, mouthful, delicate, and subtle. Even some scientists tended to not recognize umami as a basic taste. In the 1990s, umami was accepted as a basic taste by the academia. Chaudhari et al. [5] and Li et al. [6] found umami receptors on the tongue, and umami started to receive attention from many fields. It is known that the effect of umami can be enhanced by the combination of glutamate and nucleotides (inosinate or guanylate). In 2013, washoku (Japanese cuisine) was listed in the Intangible Heritage of UNESCO, and umami is now getting more attention from many people worldwide and is a known word worldwide.

Glutamate was first found as an umami substance from konbu (kelp). Konbu contains abundant glutamate; nevertheless, other food materials such as cheese, green tea, and tomato are also rich in glutamate. Human breast milk has high glutamic acid content as well [7], which means that umami is the first taste experienced by newborn babies. This component of umami is found in various traditional foods worldwide and contributes to many culinary cultures. In fact, soy sauce and miso are rich in glutamate and are very popular in Japan. There are

many types of traditional umami seasonings in other countries as well. In Southeast Asia, fish sauce (nam-pla in Thailand, nuoc mam in Vietnam, patis in the Philippines) and shrimp paste (belachan in Malaysia, terasi in Indonesia, bagoong in the Philippines, kapi in Thailand) are very popular. Fish sauces were also used more than 2,500 years ago in the ancient Greece (as garon) and ancient Rome (as garum) [8]. In the USA, ketchup made from tomatoes is also a type of umami seasoning. Vegemite is a food unique to Australia, and it is made from yeast extract.

Glutamate was commercialized in 1909 as umami seasoning. The estimated market is 2.6 million tons per year (2011), and it is still growing at a rate of 4–5%. At present, it is sold in more than 100 countries. It is used not only in Japan but also in other Asian countries and other parts of the world. They use glutamate for their own cuisine. Because of the invention of the fermentation method, the output volume of MSG had increased 100 times from 1950 to 1970. Glutamate fermentation has become essential in the modern world (Fig. 3).

There are many people who still doubt the safety of MSG. The doubt originated from the report of an American scientist, Dr. Olney [9]. In his experiment using neonatal mice, large amounts of MSG were administered by injection or were forced through direct tube. This resulted in brain damage in these animals. However, Dr. Takasaki conducted another experiment. He used pregnant, lactating, or weaning mice; the animals were fed MSG with food or with water. In his experiments, no pathogenic change was observed [10]. Therefore, he concluded that the consumption of MSG from normal food intake is not harmful. There is also a report about Chinese restaurant syndrome (CRS): the ingestion of Chinese dishes caused symptoms such as headache, numbness, and palpitations. Dr. Kwok proposed that MSG is one of the compounds causing these symptoms [11]. To clarify this, a double-blinded, multicenter clinical study was conducted [12]. In this study, 130 volunteers were fed food samples containing MSG or other substances. The tests were repeated several times. The study concluded that CRS is not caused by the intake of MSG. In conclusion, on the basis of the data from many studies, MSG is confirmed to be safe by international organizations. The Joint Expert Committee for Food Additives (JEFCA) has confirmed the safety of MSG. The acceptable daily intake (ADI) is yet to be specified. Committees of the European Commission

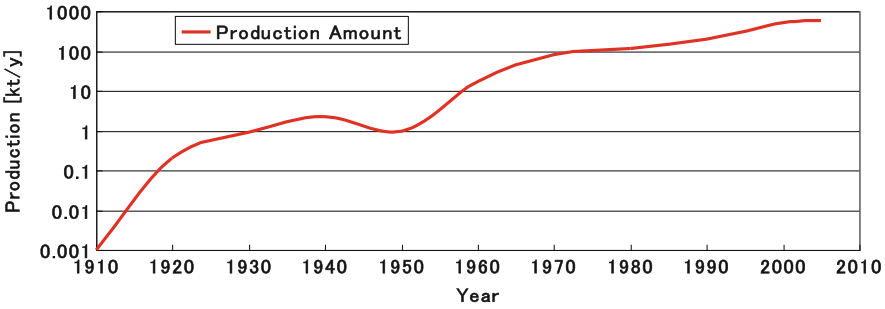

Fig. 3 Production volume of MSG producing method in days

(EC) and the USA have also confirmed the same. MSG is now one of the most extensively investigated compounds.

5 Other Food Uses

Amino acids are crucial to the taste of food. They interact with other ingredients in food to create the final taste. The taste of glutamic acid is umami. However, each amino acid has its own taste. For example, glycine, alanine, threonine, proline, and serine are sweet; phenylalanine, tyrosine, arginine, valine, leucine, isoleucine, methionine, and lysine are bitter; and glutamate and aspartate have an umami taste. Various combinations of amino acids are essential to determine the taste of food. For instance, glutamate, glycine, alanine, and arginine are essential for the flavor of crab or scallop. For the flavor of sea urchin ("uni" in Japanese dishes), amino acids such as glutamate, glycine, alanine, valine, and methionine are important. If methionine is removed from sea urchin, the taste will be somewhat like shrimp or crab [13]. In another case, for tomato flavor, the presence of glutamate and aspartate (at a ratio of 4:1) is essential. The removal of the glutamic and aspartic acid from the tomatoes resulted in a flavor resembling that of fresh cranberries.

Aspartame, an aspartic acid–phenylalanine methyl ester, a kind of peptide, is used as a low-calorie sweetener. Although carbohydrates are essential for us as the source of energy, excess of carbohydrates in the diet is a serious problem that can lead to obesity and other lifestyle diseases. The peptide tastes very sweet; it is 200 times sweeter than sucrose. The production of aspartame (aspartic acid–phenylalanine methyl ester) as a sweetener was first commercialized in 1982, although an affordable way of production was discovered in 1965. This product is currently used in over 120 countries worldwide.

6 Animal Nutrients

If we eat beef, will we turn into a cow? Of course not. Well, why not? This is because proteins are not absorbed into the human body directly. Instead, they are broken down into amino acids. Amino acids are absorbed in the intestine and transported throughout the body. Following this, amino acids are reconnected in the body to form human proteins.

In livestock industry, feed is used to supply energy and as building blocks of the body. For the building of the animal body, a good balance of each essential amino acid is important. However, the amino acid content of most feed ingredients such as corn or wheat is not well balanced. If we use only corn or wheat to feed the animals, most amino acids will not be used to build the animal body but will be wasted in excrements. On the other hand, soybean has a good balance of amino acids; however, the yield per area is low. It is possible to make corn into a well-balanced

feed by supplementing it with amino acids such as methionine, lysine, threonine, and tryptophan. Methionine is supplied as the chemically synthesized DL-methionine because it is metabolized in the animal body and used as L-methionine. Lysine, threonine, and tryptophan are produced by fermentation, and the estimated market (in 2012) was 2.0 million tons for lysine, 0.33 million tons for threonine, and 9.0 thousand tons for tryptophan. For example, when we fed 35-kg pigs with feed supplemented with amino acids, they grew faster than pigs fed by feed only, which is insufficient in amino acids [14]. Weight gain of the pigs for 4 weeks in well-balanced amino acid feed group was 26 kg, whereas that of pigs in the corn feed group was only 8 kg.

The demand for farmland has been increasing with the increase in global population. The development of new farmland, however, harms the environment through deforestation. Feed-use amino acids can be used to replace the soybean meal in the conventional corn feed without changing the amino acid balance of the feed. The yield of corn per unit land is approximately 3 times higher than that of soybeans. Therefore, this replacement will lead to more effective use of farmland. Furthermore, amino acids can reduce the environmental impact of the livestock industry. One of the biggest issues in the livestock industry is its increasing environmental impact due to the animal excrement which contains nitrogen compounds. These compounds give rise to ammonia and nitrogen oxide, causing air, soil, and groundwater pollution. As the well-known "barrel theory" proposes, in a poorly balanced feed, most amino acids are not used for the growth of livestock but are excreted. However, by adding amino acids to produce a well-balanced feed, the amino acids are efficiently used for growth. And as the result, the environmental impact of the animal excrement is decreased. The use of amino acid supplements can contribute to the reduction of environmental issues worldwide.

7 Pharmaceuticals

The nutritional functions of amino acids are also important in the medical field. Patients with mouth damage or accepted for a surgery are sometimes unable to receive sufficient nutrition through food consumption. In order to continue living, they must replenish that lost nutrition. In 1956, an infusion containing highly purified amino acids was first commercialized. At that time, infusions often had adverse side effects caused by the impurities in amino acids. Most of these problems were avoided using highly purified amino acids. Since then, various products with purified amino acids of medical grade have been supplied, e.g., medical foods, through the gastrointestinal tract or intravenously. Today, the world market for the medical-use amino acids is more than 30,000 tons per year.

In particular, branched-chain amino acids (BCAAs) are widely used. They can improve the nutritional status even in patients with decompensated cirrhosis, a serious liver disease. Hepatitis is the inflammation of the liver, and it can progress to cirrhosis if left untreated; cirrhosis can progress further to liver failure or liver

cancer. Pharmaceuticals containing BCAAs directly act on liver cells to promote protein synthesis. By assisting the function of the liver, these pharmaceuticals improve the patient's nutritional status and slow down disease development.

Because many studies on the functions of amino acids are in progress, the use of amino acids will increase in the medical field in the future.

8 Cosmetics

Recently, amino acids have attracted more attention in the cosmetics market. The estimated market of amino acids for use in cosmetics is 14,000 tons per year (2012). Skincare is an important field in cosmetics. The world market of cosmetics is USD 230 billion, of which USD 50 billion is accounted for by skincare products. Amino acids are also used for skincare. Collagen is the main protein present in the skin; it keeps the skin elastic by forming a flexible mesh structure. Exposure to UV rays or aging can break this mesh, causing wrinkles and sagging. A diet rich in proteins with good amino acid balance is effective to maintain beautiful skin. On the other hand, the surface layer of the skin has an important function of limiting the loss of water (Fig. 4). At the surface, natural moisturizing factors (NMFs) are the key to this functionality [15]. Approximately half of NMFs are free amino acids [16] and pyrrolidone carboxylic acid (PCA), which is a metabolite obtained from glutamate [17]. Using lotions or creams enriched with amino acids will efficiently improve the skin moisture content [18].

As described above, amino acid derivatives are used for foaming cleansing agents. In particular, N-acyl glutamate, which is produced by the reaction of glutamate and a fatty acid obtained from palm oil, is known to be ultra-mild for the skin and hair; it is also environment friendly. It causes lesser skin irritation and has higher biodegradability than the regular soap. N-acyl glutamate is widely used

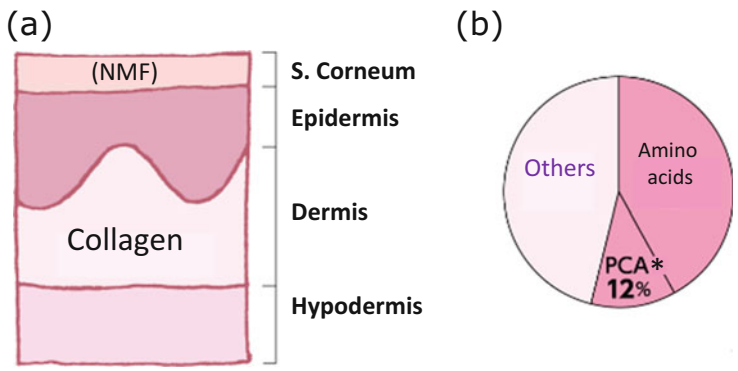

Fig. 4 Amino acids on human skin. (**a**) Structure of skin. (**b**) Composition of natural moisturizing factor (NMF). **PCA* pyrrolidone carboxylic acid

in many body shampoos for individuals with sensitive skin and as the base for baby soap [19].

Amino acids are also used in the haircare field. There are many causes of damaged hair, such as friction due to brushing or washing, heat from hair dryers, UV light, and chemical treatments such as dyeing or permanent waving. Over 80% of our hair consists of proteins. Various factors can cause the degradation of the proteins, and the hair gradually loses the proteins, resulting in dry, unruly hair or split ends. Amino acids play a role in protecting hair from further damage, replenishing the voids created by the loss of proteins and moisturizing the hair.

Cysteine is the most abundant amino acid contained in the hair proteins. The formation of disulfide bonds (S–S bonds) with other protein chains is responsible for the toughness of the hair. Cysteine and its derivative, N-acetyl cysteine, have strong reductive properties conferred by the thiol residue. They are used in the reduction reagent for permanent wave. The mechanism underlying permanent wave is cutting the disulfide bonds using the reduction reagent, followed by reforming the bonds (with a different protein chain) using the oxidation reagent. A chemical reduction agent such as thioglycolic acid (TGA) is often used; however, cysteine has the advantage of reducing the hair damage and is suitable for the formation of soft waves [20]. Cysteine has lower acute phase toxicity and causes lesser skin irritation than TGA.

9 Conclusions

The first amino acid to be commercialized was MSG, which was purified from a gluten lysate. Later, the amino acid industry started to use the residual of gluten lysate. This development of the use of each amino acid expanded with the discovery of the functions. On the other hand, the supply of these amino acids at a low price is very important for industrial applications. Undoubtedly, the invention of the glutamate fermentation process is an epoch-making event. It has made it possible to produce large amounts of glutamate at a low price. In fact, the production volume increased 100 times in the 20 years after the invention. Nevertheless, the development of fermentation methods for other amino acids is equally important. In particular, with the increase in meat consumption in Asia and Central and South America, the production volume of the feed-use amino acids is rapidly increasing. The production volume of lysine is currently at a similar level as that of glutamate. Many new functions of amino acids are extensively investigated at present, and the resulting functions are expected to expand the fields of the amino acid industry. These new functions and possible application of amino acids are described in Chap. 13.

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Discovery and History of Amino Acid Fermentation

Shin-ichi Hashimoto

Abstract There has been a strong demand in Japan and East Asia for L-glutamic acid as a seasoning since monosodium glutamate was found to present umami taste in 1907. The discovery of glutamate fermentation by *Corynebacterium glutamicum* in 1956 enabled abundant and low-cost production of the amino acid, creating a large market. The discovery also prompted researchers to develop fermentative production processes for other L-amino acids, such as lysine. Currently, the amino acid fermentation industry is so huge that more than 5 million metric tons of amino acids are manufactured annually all over the world, and this number continues to grow. Research on amino acid fermentation fostered the notion and skills of metabolic engineering which has been applied for the production of other compounds from renewable resources. The discovery of glutamate fermentation has had revolutionary impacts on both the industry and science. In this chapter, the history and development of glutamate fermentation, including the very early stage of fermentation of other amino acids, are reviewed.

Keywords Amino acid fermentation, *Corynebacterium glutamicum*, Glutamate fermentation, L-Amino acid, Metabolic engineering, Strain breeding

Contents

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Amino acid fermentation is a huge industry. More than 5 million metric tons of amino acids (more than 2 million metric tons of glutamate, around 2 million metric tons of lysine, etc.) are manufactured annually all over the world [1]. Amino acids find application in a wide range of fields: seasoning, animal feed, medicine, and starting material for chemicals including pharmaceuticals. A number of scientific paper and patent on amino acid fermentation have been published every year. These studies have been contributing to widen our knowledge on the science of metabolism, genetics, and physiology.

The discovery of glutamate fermentation by *Corynebacterium glutamicum* in 1956 was the inception of amino acid fermentation. This discovery was a major breakthrough not only for glutamate production but also for research, demonstrating that amino acid fermentation is possible. In this chapter, the history of amino acid fermentation (glutamate fermentation is mainly focused on) before and after its discovery is reviewed.

1 Prehistory of Glutamate Fermentation

Although glutamate was isolated from gluten in 1866, its major applicational value was found in 1908 by Kikunae Ikeda [2]. He studied taste presenting substance derived from kelp, whose soup has traditionally been used as a basic seasoning in Japan, and identified the monosodium salt of L-glutamate as an *umami* substance. An entrepreneur, Saburosuke Suzuki, collaborated with Dr. Ikeda and commercialized the discovery; he began selling monosodium glutamate (MSG) as a new seasoning "AJI-NO-MOTO®" in 1908 (Fig. 1).

Because umami is a traditional taste component in Japan, the new product got a great sales success. However, there were several difficulties in manufacturing MSG, for which wheat gluten was hydrolyzed using hydrochloric acid and L-glutamate was isolated following the recrystallization as the monosodium salt. Because there were scarce materials that could tolerate acidic conditions under high temperature during hydrolyzation in the early twentieth century, obtaining suitable vessels for hydrolyzation was a big issue. After several trials and errors, a certain type of ceramic pot was found to be suitable. Hydrogen chloride gas released during the hydrolyzation process was very hazardous. Furthermore, the

Fig. 1 The first product of umami seasoning, "AJI-NO-MOTO®." The photo is reprinted under the kind permission of Ajinomoto Co., Inc.

formation of a large volume of waste (the remaining fraction of the hydrolyzate) presented additional issue [3].

Therefore, there was a strong demand for a new manufacturing process for MSG or L-glutamate. During the mid-1950s, a decade after the end of World War II, the economy and life of people in Japan was returning to normal. Corresponding with the social situation, the demand for MSG had increased rapidly; the production of MSG by Ajinomoto Co., Inc jumped from 6,662 metric tons in 1955 to 13,586 metric tons in 1959 [4].

Several attempts were made to establish a new method for manufacturing L-glutamate. Because of the necessity of optical resolution, chemical synthesis was not advantageous while it was used commercially for a particular period. Chemical synthesis coupled with enzymatic resolution was a feasible method [5]. Izaki

et al. have reported specific degradation of D-glutamate by *Aerobacter* [6]. The optical resolution of *N*-acyl-DL-glutamate by D-specific acylase activities derived from *Aspergillus tamarii* and *Penicillium vinaceum* presented additional approach [7].

Since glutamate is biosynthesized from 2-oxoglutarate through amination, it was natural to pursue the process that consisted of microbial production of 2-oxoglutarate and microbial amination of the oxoacid.

The earliest report on fermentative production of 2-oxoglutarate can be found in 1946; *Pseudomonas fluorescens* accumulated 16–17 g of the acid per 100 g of glucose [8]. The yield was increased to 41 g of the acid per 100 g of glucose [9].

Formation of small amounts of glutamate in the presence of 2-oxoglutarate and ammonium salts was reported in *Clostridium* [10], *Escherichia coli* [11], *Bacillus subtilis* [12], *P. fluorescens* [13], and *Pseudomonas ovalis* [14]. Attempts to perform the conversion using amino acids as amino donors were also reported [13, 15].

The combination of 2-oxoglutarate fermentation and amination of the oxoacid appears to be just one step away from direct fermentation of glutamic acid (direct fermentation refers to the process of producing the product from a low-cost carbon source, such as sugar, and a nitrogen source, such as ammonia, through the cultivation of microorganisms). It was known that accumulation of small amounts of glutamate occurred in the cultivation medium of certain bacteria. Morrison and Hinshelwood and Dagley et al. have observed very small amount of accumulation of the amino acid in the cultivation broth of *E. coli* and *Aerobacter aerogenes* [16, 17]. Thorne et al. have reported that *B. anthracis* formed glutamate (2 g/L) under the conditions in which polyglutamate formation was hampered [18]. Asai et al. screened for a glutamate producer and found *Micrococcus varians* to be the best producer although the accumulation was far lower (2.9 g/L at the optimal efficiency) for industrial application [19].

2 Discovery and Industrialization of Glutamic Acid Fermentation

M. J. Johnson, an emeritus professor at the University of Wisconsin, described the situation in 1955, “there is, in many quarters, great interest in fermentative glutamic acid production” [20]. However, there was a strong notion that it is irrational to expect a microorganism to accumulate a large quantity of the amino acid extracellularly because (1) glutamate is an essential component for the organism, (2) excretion of glutamate is an economical loss for the cell because the biosynthesis of the amino acid is energetically expensive, and (3) secretion of glutamate would not be expected to have suppressing effect against surrounding microorganisms like antibiotic dose. Researchers in Kyowa Hakko Kogyo Co., Ltd. have overcome this conceptual obstacle.

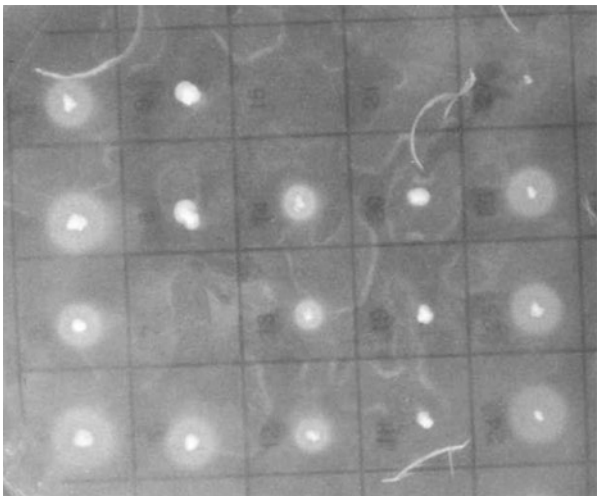

Fig. 2 Bioassay screening of glutamate-producing microorganisms. Glutamate productivity of the test strain can be estimated by the scale of the halo formed around the strain. The photo is reprinted under the kind permission of Kyowa Hakko Bio Co. Ltd.

Dr. Udaka, a researcher of Kyowa Hakko Kogyo Co., Ltd. at that time, has set the following screening method [21]. Bacteria isolated from the environment were replicated on nutrient agar plates and several types of defined medium plates (test plates). After colony formation, the test plates were exposed to UV radiation to kill the organisms. Then, soft agar medium containing the glutamate auxotroph *Leuconostoc mesenteroides* was overlaid on the test plates. The halo of growth development around the colony on the test plates indicated glutamate excretion by the colony (Fig. 2).

After screening *only* approximately 500 isolates, they found the superior strain, *Corynebacterium glutamicum* (originally reported under the name of *Micrococcus glutamicus*). The strain accumulated 10.3 g of glutamate per liter when cultivated in a flask with liquid synthetic medium with 5% glucose [22], and the accumulation was easily increased to >30 g/L [23] with >25% yield against glucose input, indicating that the strain is applicable for industrial glutamate fermentation.

When the study was scaled up, however, the researchers faced a puzzle; the bacterium produced only trace amounts of glutamate. Several months of intensive research revealed important characteristics of glutamate fermentation: the bacterium is a biotin auxotroph and produces glutamate only under biotin-limited conditions. It was assumed that the cells grew in screening and flask cultivation using a trace amount of biotin carried over from the pre-culture and, thus, resulted in biotin-limited conditions.

The first commercial fermentation of glutamate was conducted in 1958 at a plant of Kyowa Hakko Kogyo. There were still problems to be solved for commercialization, such as the downstream process, particularly the control of the crystal form. In that way amino acid fermentation took the first step.

3 Establishment of Glutamate Fermentation

Glutamate fermentation by *C. glutamicum* was first presented at the International Symposium on Enzyme Chemistry held in Japan in 1957. As the congress was the first major international scientific meeting held in Japan after World War II, the presentation attained great attention from Japanese researchers in the field of life sciences. Once the discovery of glutamate fermentation was public, several researchers and companies rushed into the research. It looks like a "gold rush," which is observed in Figs. 3 and 4.

Until 1970, Japanese researchers enthusiastically conducted research and held a monopoly on the scientific papers on glutamate fermentation (Fig. 3). Japanese research activity appeared to steady down by the 1980s; however, it showed resurgence during the 2000s. In contrast, reports from other Asian countries, particularly from China and India, showed an increase from the 1980s. Several of these studies have outlined the discovery of a new strain (most strains are under *Corynebacterium* sp.), the application of a new raw material, and improvement of the production system, suggesting a strong economical demand of glutamate fermentation in these areas. Research from Europe began to emerge from the 1990s. Most of the European research focused on the mechanism of glutamate fermentation and played an essential role in understanding the mechanism.

Figure 4 shows the "gold rush" in Japan from a different angle. A rapid increase of patents in 1960 suggests that companies began research on glutamate fermentation after the announcement of the discovery of *C. glutamicum*. Representative strains and carbon source are shown in Table 1. Most of the important aspects of the fermentation appeared before 1980, which are summarized below. These insights are closely or directly related to the elucidation of the mechanism of glutamate production discussed in [35].

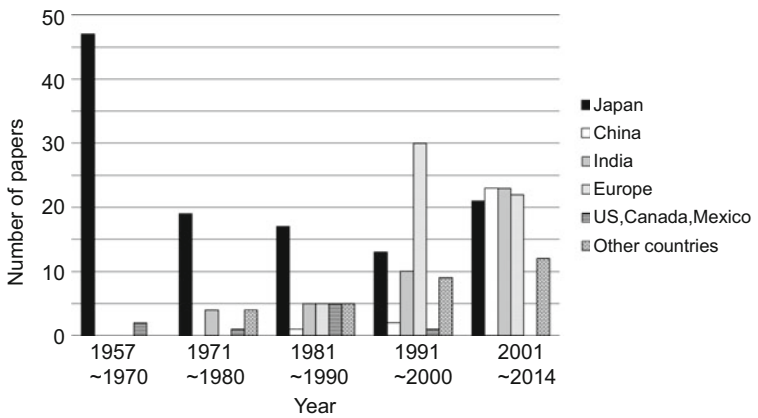

Fig. 3 Trends of scientific paper publication on glutamate fermentation in English. For each decades (except for 1957–1970 and 2001–2014), the number of papers are counted by country. Reviews, patents, and oral presentations are not included

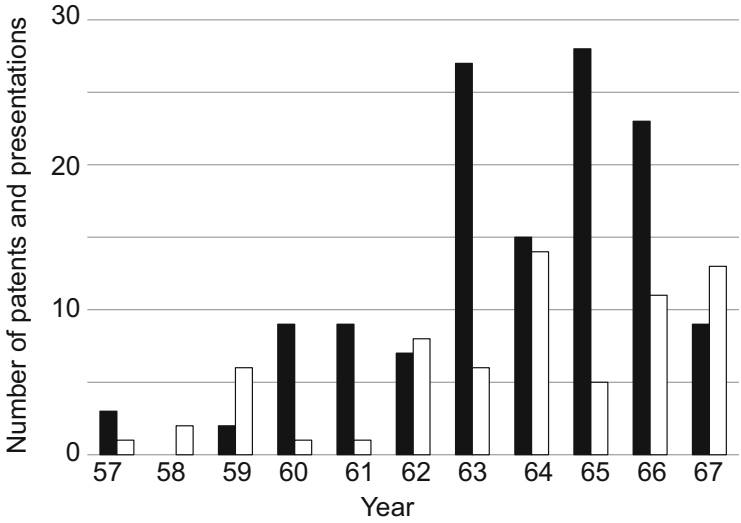

Fig. 4 Numbers of Japanese patents (*black bar*) and oral presentations (*white bar*) in the annual meeting of Japan Society for Bioscience, Biotechnology, and Agrochemistry from 1957 to 1967

Table 1 Glutamate fermentation from different carbon sources by different microorganisms

Carbon source	Microorganism	Titer (g/L)	Reference
Glucose 12%	*C. glutamicum*	30	[24]
Glucose ?%	*C. glutamicum*	195	[25]
Cane molasses 13%	*C. glutamicum*	63	[26]
Acetate 9%	*C. glutamicum*	23	[27]
Ethanol ?%	*Brevibacterium* sp.	53.1	[28]
Methanol 11%	*M. methylovora*	6.8	[29]
Methanol ?%	*B. methanolicus*	69	[30]
n-Paraffin 3%	*Corynebacterium* sp.	5	[31]
n-Hexadecane 8%	*C. hydrocarboclustus*	19.6	[32]
Benzoate ?%	*Brevibacterium* sp.	75	[33, 34]

The earliest scientific report (patents and oral presentations are not included) on each carbon source is listed. For glucose and methanol, reports of the highest titer are also shown. Symbol "?" in the carbon source indicates no description of the amount of input carbon source in the report

3.1 Producer Strain

Many species were claimed as glutamate-producing microorganisms such as *Brevibacterium lactofermentum*, *Brevibacterium flavum*, *Corynebacterium callunae*, *Corynebacterium lilium*, etc. Most of them were aerobic, gram-positive, nonacid-fast, nonspore-forming, rod-shaped, and biotin-requiring bacteria. These "new" species were categorized in the spices of *Corynebacterium glutamicum* by

thorough taxonomic investigation in later years [36–38]. Thus, the name of *C. glutamicum* is used for these bacteria hereinafter. It became a shared sense within several years after the discovery of the bacterium that the bacterium (even wild-type strain) produces glutamate at a yield of >40% against input sugar (Table 1) under appropriate conditions.

Bacteria capable of producing glutamate from sugar and not belonging to *C. glutamicum* have also been reported. Chao and Foster have reported productivity of 13.5 g/L from 3% glucose by *Bacillus megaterium* [39]. It is interesting that this strain is also biotin auxotroph. Some *Arthrobacter* [40] and *Streptomyces* [41] strains were also reported to produce glutamate. Recently, it was reported that metabolically engineered *E. coli*, *Enterobacter agglomerans*, *Klebsiella planticola*, and *Pantoea agglomerans* produce significant amounts of glutamate [42, 43].

3.2 Carbon Source

As *C. glutamicum* readily utilizes glucose, fructose, and sucrose, these sugars are initially used as a carbon source. Molasses, a more economically desirable source, was mainly used after the discovery of an alternative method of biotin limitation because it contains excess amount of biotin. Ethanol and acetic acid have also been reported to provide the bacterium a good productivity. Recently, it was demonstrated that the spectrum of usable sugar of *C. glutamicum* can be expanded by expressing heterologous genes [44].

Carbohydrate obtained by petrochemistry was studied as an alternative carbon source. Shiio and Uchio have reported several kinds of bacteria capable of forming glutamate from paraffin [32]. *C. hydrocarboclustus* was reported to have relatively high productivity (Table 1). Ghosh and Banerjee have described the production from n-alkane by the *Serratia marcescens* strain [45]. In addition, aromatic compounds, such as benzoate, have been investigated as a carbon source [33, 34, 46].

Glutamate fermentation from methanol has been attempted since the 1970s [29, 47]. As *C. glutamicum* cannot assimilate methanol, various microorganisms such as *Methanomonas methylovora* [29], *Methylobacillus glycogenes* [48], and *B. methanolicus* [30] have been screened and used. The *B. methanolicus* strain M168-20(pHP13) appears to be the highest producer from methanol (Table 1). Glutamate production through photosynthesis (carbon source is CO_2) has also been investigated using algae [49, 50].

3.3 Production Conditions

As described above, biotin limitation was the crucial factor of glutamate fermentation by *C. glutamicum*. This feature made it impossible to use some economically desirable raw materials, such as molasses. Attempts to clarify the underlying

mechanism and finding alternative methods of biotin limitation have been made since a very early period. Shiio et al. first reported that biotin limitation causes the change of cellular permeability of amino acids including glutamate [51]. Reports that followed confirm the association between biotin limitation and glutamate permeability [52, 53].

Several alternative methods of biotin limitation were devised in the 1960s. One of the major breakthroughs was the addition of penicillin; adding an appropriate amount of penicillin at an early stage of cultivation triggers glutamate production under biotin excess conditions [54]. Addition of surfactant was an alternative method. Because the effect of surfactant depends on its chemical composition, specific surfactants were chosen, such as polyoxyethylene sorbitan monostearate [55, 56] and cetyltrimethylammonium bromide [57].

Penicillin inhibits cell wall synthesis. The first action point of a surfactant should be the cell surface. Putting these and the biotin effect together, the "permeability hypothesis" or the "leak model" was claimed to be the mechanism of glutamate fermentation; change in cellular permeability of glutamate caused by some treatment triggers the leak out of the amino acid, which shifts intracellular metabolism toward glutamate formation [53, 58]. The finding of the relationship between fatty acid composition in the cell membrane and glutamate productivity [59, 60] supported the notion. It was also supported by the finding that an oleic acid-requiring mutant produces glutamate under biotin-sufficient conditions [26, 61]. Based on these lines of evidences, the mechanism of glutamate fermentation seemed to have been settled around 1970. However, further contention emerged later (see below and [35]).

Conditions other than biotin were investigated in detail, but only effects of oxygen and pH are mentioned here. Glutamate production by *C. glutamicum* requires aerobic conditions. Under oxygen-insufficient conditions, the bacterium produces succinic acid and/or lactic acid [62]. Medium pH should be maintained slightly above 7.0. When pH is controlled to be acidic, around pH 5.5, the bacterium produces mainly glutamine.

4 Recent Development in Glutamic Acid Fermentation

4.1 In C. glutamicum

In general, (a) genetic modification(s) is necessary for forcing a microorganism to overproduce a certain amino acid, for example, deregulation of aspartokinase for lysine production. Glutamate production by *C. glutamicum* is different. The wild-type strain exerts high productivity under appropriate conditions as described above. Although there have been a lot of patents claiming mutations beneficial for glutamate production by the bacterium, none of them appear to improve the productivity significantly. However, since the 1990s, genes essential for glutamate

fermentation have been identified, which shed new light on the mechanism. Since the mechanism is discussed in Chap. 4, a very rough sketch is presented below.

There have been two predominant hypotheses on the mechanism. The first idea is the "permeability hypothesis" as described above. The other is the "flux hypothesis," claiming that change in carbon metabolism is the major cause of the overproduction, which is a quite common concept among other amino acid fermentations. Facts supporting each hypothesis are presented below; however, it should be noted that the two hypotheses are not necessarily exclusive.

Before mentioning on the flux hypothesis, the biosynthetic pathway of glutamate is briefly reviewed. Glutamate is formed by amination of 2-oxoglutarate. Glutamate dehydrogenase (GDH) and the coupled reactions of glutamine synthetase and glutamine synthase (GS/GOGAT system) are involved in the amination [63–67]. While cell growth can be sustained either by GDH or GS/GOGAT system, GDH is responsible for glutamate overproduction [67]. Carbon from sugar is metabolized to 2-oxoglutarate through glycolysis and part of the TCA cycle. Enzymes contributing to the flux have been clarified, although the flux around oxaloacetate is complicated. Characteristics and regulation of these enzymes have also been reported [68–73]. 2-Oxoglutarate is the important branch point, the glutamate-forming direction by amination and the glutamate-degrading direction by oxidative decarboxylation to succinate with 2-oxoglutaratedehydrogenase complex (ODHC). ODHC is the focal point in flux control.

During the 1960s, it was assumed that *C. glutamicum* had no or very low activity of ODHC [74]. This was the basis of the flux model during the early 1960s, but the permeability hypothesis became dominant as described above. Later, it was confirmed that *C. glutamicum* possessed certain ODHC activity [75]. The flux hypothesis was revived by the finding that deletion of the *odhA* gene (encoding E1o subunit of ODHC) conferred the bacterium glutamate overproductivity ([76], oral presentation was in 1996). Simultaneously it was reported that ODHC activity is reduced under glutamate-producing conditions [77]. Kim et al. have reported the supportive results [78]. Recently, it was revealed that ODHC activity is regulated by OdhI/PknG [79] and that OdhI, the inhibitor of ODHC, is induced under glutamate-producing conditions [80].

In parallel with the revival, supportive findings for the permeability hypothesis have also been accumulated. In 1989, Hoischen and Kramer have demonstrated by biochemical analysis that glutamate excretion is mediated by an active efflux system and not by simple leakage [81]. They further demonstrated the importance of alternation of membrane tension [82, 83]. Changes in the membrane components cause such alternation of membrane tension, and there have been several reports indicating the association between change in membrane composition and glutamate overproductivity [84–86].

Genetic findings supporting the permeability hypothesis have also been accumulated. Kimura et al. have described the *dtsR* gene that restores detergent (Tween 40) sensitivity [87]. DtsR has a high similarity to the β-subunit of methylmalonyl-CoA carboxyltransferase, and it is suggested to be the biotin enzyme involved in fatty acid biosynthesis. The disruption of *dtsR* causes oleic acid auxotrophy and

glutamate production [88]. The *ltsA* gene was found to code a gene whose disruption causes the cell to be lysozyme sensitive and glutamate productive [89, 90]; however, the physiological role of the gene product is not known. Nakamura et al. found a mechanosensitive channel Ncgl1221 for glutamate excretion [91–95]. Mutation of the gene caused glutamate overproduction even in the presence of intact OdhA [92, 94].

The production mechanism still remains elusive, but it will be comprehensively understood in the near future.

Because glycolysis and the TCA cycle are the major carbon metabolic pathways to glutamate, the relation between energy metabolism and glutamate production is a point to be investigated. In this respect, Yokota et al. have reported that mutants decreasing H-ATPase activity at below 1/4 the level of the wild type produce less glutamate [96].

4.2 In Other Bacteria

It was a strong support of the flux hypothesis that the disruption of *odhA* confers *E. coli* glutamate productivity [43, 74]. Since glutamate productivity by the disruption of the gene has known in other bacteria [42], at least, reducing the flux through ODHC appears to be generally applicable.

Glutamate excreted in the medium is reimported by the glutamate import system in *C. glutamicum* [97, 98] or other bacteria, such as *E. coli* [99]. Thus, an increase in glutamate in the medium exerts a negative effect on glutamate synthesis through the regulation of biosynthetic enzymes. When glutamate dissolved in the medium forms crystals, it no longer has further effect on the regulation. Since isoelectric point of glutamate is around pH 5.5, glutamate concentration dissolved in the medium is lowest at the pH area. Thus, if a bacterium that grows and produces glutamate in this pH, it must be advantageous for glutamate production by reducing the negative effect of glutamate in the medium. Based on this idea, several bacteria were found and constructed as the producers [100]. The entire genome sequence of one strain has recently been published [101].

5 Amino Acid Fermentation

Whether it is a natural expectation or not, researchers in Kyowa Hakko Kogyo Co., Ltd. thought that *C. glutamicum* might produce other amino acids immediately after the discovery of glutamate fermentation. They started delivering mutants for amino acid producers. The attempt quickly resulted in the second amino acid fermentation; a mutant requiring arginine accumulated ornithine [24]. This was the first report releasing regulation on amino acid biosynthesis by amino acid auxotrophy. Further, in the next year, the researchers reported lysine-producing mutants of

C. glutamicum based on the same idea [102]. Earliest reports on each of the amino acid fermentations are listed in Table 2.

The other important method liberating regulation is delivering analogue-resistant mutants. Although it was known since the 1950s that analogue resistance confers amino acid overproductivity [106, 108], the first clear and quantitative example was presented by Sano and Shiio [103]. They demonstrated that an *S*-(2-aminoethyl)-L-cysteine-resistant mutation makes aspartokinase of *C. glutamicum* insensitive to feedback regulation by lysine, resulting in lysine production. Since obtaining resistant mutants (positive screening) is easier than obtaining auxotrophic mutants (negative screening), this method was a very powerful tool for amino acid fermentation.

Producer breeding was not limited to *C. glutamicum*. Other bacteria, such as *E. coli* and *S. marcescens*, were manipulated and used as amino acid producers. In these enterobacteria, destroying the degrading activity of the target amino acid was important at some times. For example, the construction of the threonine producer of *S. marcescens* was initiated by getting a mutant incapable of metabolizing threonine [116]. Destruction of the amino acid-degrading ability and deregulation of the biosynthetic pathway can be regarded as the general strategy of strain improvement.

Table 2 Earliest scientific reports on each of the amino acid fermentations

Amino acid	Type of producer	Titer (g/L)	Reference
Ornithine	*C. glutamicum* A-mutant	26.2	[24]
Lysine	*C. glutamicum* A-mutant	14	[102]
	C. glutamicum R-mutant	32	[103]
Tyrosine	*E. coli* R-mutant	?	[104]
Tyrosine, phenylalanine	*C. glutamicum* R-mutants	2.2	[105]
Methionine	*E. coli* R-mutant	?	[106]
	C. glutamicum A- and R-mutant	2	[107]
Histidine	*E. coli* R-mutant	?	[108]
	C. glutamicum R-mutant	7	[109]
Valine	*Aerobacter cloacae* strain isolated by screening	12	[110]
Threonine	*E. coli* A-mutant	3.7	[111]
Proline	*C. glutamicum* A-mutant	11.4	[112]
Tryptophan	*C. glutamicum* A- and R-mutant	2	[113]
Isoleucine	*S. marcescens* R-mutant	6.7	[114]
Leucine	*S. marcescens* A- and R-mutant	13.5	[115]

Patents and oral presentations are not included. For lysine, the first report of auxotrophic mutant and that of the analogue resistant mutant are shown. For methionine and histidine, the first reports describing the titer are also shown. A-mutant, auxotrophic mutant; R-mutant, analogue resistant mutant. Symbol "?" in titer indicates no description on the titer in the report

Since the 1980s, recombinant DNA technology has been available and applicable for strain improvement. Gene dosage effect is the most instant use of the technology. A gene coding for a key enzyme is amplified by cloning on a multicopy plasmid. An increase in the expression results in the increase of the activity, enhancing the metabolic flux to the desired direction. Combining conventional mutation and recombinant DNA technology enables the construction of a producer strain for the amino acid whose biosynthesis is under multiple and complex regulation, such as tryptophan [117].

Discovery of an amino acid exporter was noteworthy. The lysine exporter in *C. glutamicum* was first predicted using biochemical analysis [118, 119] and then confirmed genetically [120]. The finding is interesting not only because it is counterintuitive but also because it raises a new key concept in amino acid fermentation. Currently, over eight kinds of amino acid exporters have been identified in *C. glutamicum* [121, 122] and *E. coli* [123–127].

Knowing carbon flux in the producer microorganism is important for strain improvement. Carbon distributions between the hexose monophosphate pathway (HMP) and the pentose monophosphate pathway (PPP) and around the oxaloacetate supply have been the focal points of research interest. ^{13}C-NMR analysis combined with metabolic balance analysis has been widely used for this purpose, and much insight has been accumulated [128–130]. One of the earliest finding of the analysis is that the carbon distribution through HMP and PPP in *C. glutamicum* is different between the glutamate producer and the lysine producer [131]. The carbon flux distribution between HMP and PPP was 8:2 in the glutamate producer, whereas it was 4:6 in the lysine producer, suggesting that the flux is controlled by the necessity of NADPH. Subsequently, this finding led to cofactor engineering [132–134].

6 Feature Prospective

At the end of the twentieth century, the whole genome sequence determination was initiated in various kinds of organisms including *E. coli* [135] and *C. glutamicum* [136]. Genome data enabled a new way of strain improvement termed as "genome breeding" [137–139] that creates a minimal set of mutations beneficial for production by (1) comparative analysis of whole genomes of the wild-type strain and a producer strain obtained from the wild-type via successive classical mutations, (2) defining beneficial mutations, and (3) assembling them in the wild-type background. This enables the construction of a producer strain with high productivity without undesirable traits of the classical producer mutant, such as slow growth and stress sensitiveness.

Glutamate fermentation has been used mainly for production of MSG, a seasoning. However, it may take part in wider areas of application because glutamate is expected to be one of the basic chemicals from renewable resources [140]. Thus, glutamate fermentation yet retains enough potential for contributing for the quality

of human life in addition to its historical role in modern fermentation industry and applied microbiology.

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Early History of the Breeding of Amino Acid-Producing Strains

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Abstract Amino acid production started in Japan in 1908 with the extraction of monosodium glutamate (MSG) from acid hydrolysates of proteins. In addition to extraction, other methods of amino acid production include chemical synthesis, fermentation, and enzymatic synthesis both for glutamic acid and other amino acids. In this chapter, we review the historical transition of these production methods; currently, fermentation is the chief production method of amino acids. All wild-type microorganisms possess a negative feedback control system (feedback inhibition and repression) on the enzymes within the amino acid biosynthetic pathways. Therefore, techniques for the development of amino acid-overproducing strains and also for the establishment of enzymatic processes for the synthesis of amino acids were developed to artificially release these feedback controls. The key techniques used to bypass these controls are as follows: (a) artificial acceleration of the easy efflux of intracellularly synthesized amino acids outside of cells; (b) limitation of the concentration level of feedback inhibitors (amino acids) using auxotrophic mutants; (c) genetic desensitization of key enzymes to feedback inhibition by mutation and selection of amino acid analog-resistant mutants; (d) amplification of genes coding for desensitized biosynthetic enzymes; (e) disruption of amino acid degradation activity; and (f) application of enzyme reactions free from feedback controls for amino acid synthesis. Selection and breeding of amino acid producers by the application of these techniques is described.

Keywords Analog-resistant mutant, Desensitization, Feedback inhibition, Production method, Release of metabolic regulation, Repression

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1 Historical Transition of Amino Acid Production Methods

As is well known, in 1908, Kikunae Ikeda, a professor of Tokyo Imperial University, found that MSG was the *umami* substance contained in a type of kelp (*konbu* in Japanese) that had traditionally been used as soup stock in Japanese cooking (*washoku*) [1]. He then succeeded in producing MSG from acid hydrolysates of wheat or soybean proteins. Saburosuke Suzuki, the progenitor of Ajinomoto Co. Inc., further developed this production process and commercialized MSG as a seasoning under the brand name of "AJI-NO-MOTO®." The process was the first industrialization of amino acid production and continued successfully for approximately 50 years.

However, this production process had drawbacks in large-scale production, namely, the high cost of raw materials and the use of hot hydrochloric acid, which affected both the environment and the health of operators, as well as causing corrosion of the manufacturing facilities.

Thus, new approaches to solve these problems were investigated with both chemical synthesis and fermentation, including enzymatic synthesis.

Chemical synthesis of glutamic acid from acrylonitrile, carbon monoxide, and hydrogen cyanide by the application of the Strecker reaction was established and industrialized by the Ajinomoto Co. in 1960. The process was praised in those days as the first successful production of a food from "inexhaustible" petroleum. However, the process of obtaining L-glutamic acid through the optical division of its racemic form was long, and, therefore, the cost was rather higher than that of fermentation. Moreover, the concept of "chemically synthesized food" was often not to be accepted emotionally by consumers, and this chemical process was abolished in 1973.

The first successful fermentative production of L-glutamic acid was achieved by the Kyowa Hakko Kogyo Co. Ltd. (now Kyowa Hakko Bio Co. Ltd.) in 1957 through the finding of a novel glutamic acid-producing bacterium, *Micrococcus glutamicus* (later reclassified as *Corynebacterium glutamicum*), and the establishment of suitable culture conditions for this bacterium.

Thereafter, various L-glutamic acid-producing bacteria, such as those belonging to the genera *Brevibacterium*, *Microbacterium*, and others were found. All of these bacteria have similar taxonomic characteristics: each has a biotin requirement, with L-glutamic acid production occurring under biotin-limiting conditions, and each is a Gram-positive rod, does not form spores, is nonmotile, and has a narrow-range GC-content and an rRNA gene restriction pattern. Thus the common name *C. glutamicum* was assigned to these bacteria [2, 3]. In this chapter, however, conventional names are used from the sources of the original reports.

Since this discovery, the fermentative production of L-glutamic acid has been carried out successfully such that in 2010 the worldwide amount of MSG produced was estimated to be approximately 2.4 million tons per year.

Other amino acids were only by-products of L-glutamic acid at first; however, as science progressed, the nutritional and medical importance of amino acids was recognized and further production techniques were developed. Bacteria that could produce various amino acids were obtained by deriving amino acid analog-resistant mutants as well as auxotrophs from the L-glutamic acid-producing bacteria, *Escherichia coli* and *Serratia marcescens* (*S. marcescens*, which had been used by the Tanabe Seiyaku Co. Ltd. (now Mitsubishi Tanabe Pharma Corporation), is not supposed to be used today).

Enzymatic synthesis was developed through the conversion of metabolic precursors or chemical synthetic intermediates of amino acids to the corresponding amino acids using application of microbial enzymes. Bioreactors using immobilized enzymes or enzyme-containing cells contributed to improved yields by the stabilization and repeated use of enzymes.

Application of recombinant DNA and protein engineering techniques using *E. coli* systems has been in use to further improve the yields of fermentation and enzymatic synthesis since the 1980s.

An outline of the historical transition of the production methods for each amino acid is shown in Fig. 1. As shown in the figure, production methods, as a whole,

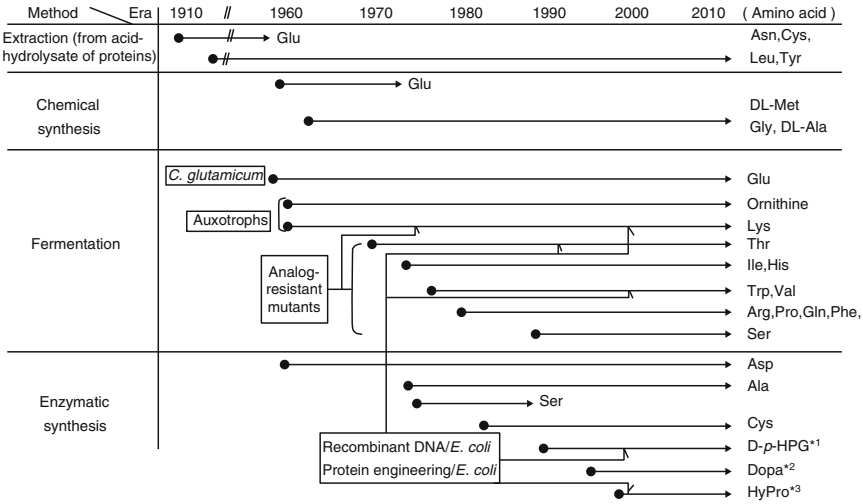

Fig. 1 Historical transition of amino acid production method. *1 D-*p*-Hydroxyphenylglycine, *2 dioxyphenylalanine, *3 hydroxyproline, ●——▶: start and period of production, □: discovery or technique

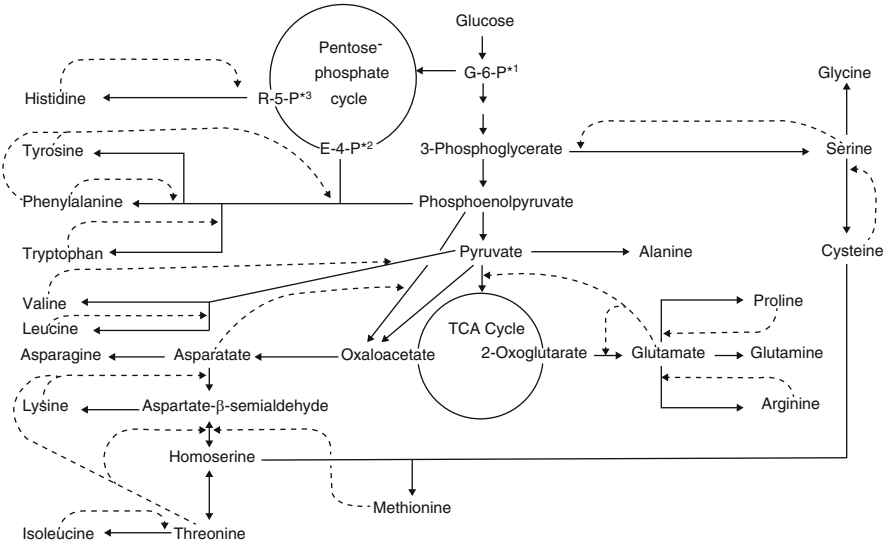

Fig. 2 Outline of amino acid biosynthesis and its regulation in microorganisms. *1 Glucose-6-phosphate, *2 erythrose-4-phosphate, *3 ribose-5-phosphate, ——▶: biosynthetic pathway, ◀- - - - - - -: feedback regulation by end product

changed from extraction from the acid hydrolysate of proteins to chemical synthesis and also to fermentation techniques, including enzymatic synthesis.

L-Tyrosine, L-leucine, L-cysteine, and L-asparagine, which had rather small markets, are produced by extraction, whereas they are produced also by fermentation today. Glycine, DL-methionine, and DL-alanine are produced by chemical synthesis. Other amino acids are the products of fermentation and enzymatic synthesis.

Thus, four methods (extraction, chemical synthesis, fermentation, and enzymatic synthesis) are all in parallel use today. Fermentation is the major production technique, yielding about 80–90% of the production quantity and 60% of the produced items.

2 Biosynthesis of Amino Acids and Its Regulation in Microorganisms

As is well known, wild-type microorganisms possess metabolic regulatory systems to prevent the overproduction of amino acids. Figure 2 shows an outline of amino acid biosynthesis and its regulation (feedback inhibition and repression) in microorganisms. Feedback inhibition works on key enzymes in the first steps of biosynthetic pathways.

Thus, it is necessary to first overcome or avoid these metabolic regulations in order to establish amino acid production. There were many pioneering works to obtain bacteria capable of production despite these regulations. For about 50–60 years, various types of amino acid production were established and industrialized, mainly in Japan, and these were commonly the result of microorganisms that were released from metabolic regulations. Techniques that were applied to overcome these regulations are classified in the following section.

3 Key Techniques to Overcome or Avoid Metabolic Regulation

3.1 Acceleration of the Efflux of Intracellularly Synthesized Amino Acids Outside of Cells

As described in Sect. 2, an overproduction of L-glutamic acid is observed when *C. glutamicum* and related bacteria are cultured in media containing either (1) limited amounts of biotin or (2) suitable amounts of penicillin or surface-active agents in media containing a high biotin concentration.

In the pathway of L-glutamic acid biosynthesis in *Brevibacterium flavum* (later reclassified as *C. glutamicum*), L-glutamic acid inhibits glutamate dehydrogenase

and represses the formation of citrate synthetase and phospho*enol*pyruvate carboxylase [4]. In spite of these negative feedback mechanisms, L-glutamic acid is overproduced, leading to the question of why this occurs. Overproduction in these culture conditions has been explained by efflux of the amino acid to the outside of the cells through the cell membrane. Due to the fact that the intracellular concentration of L-glutamic acid was lower than that of the extracellular concentration, L-glutamic acid was excreted positively, and the regulation no longer worked [5].

3.2 Limitation of the Concentration Levels of Feedback Inhibitors

If the concentration of inhibitors can be reduced to low levels, feedback inhibition of the key enzymes of amino acid biosynthesis is released. This was accomplished through use of auxotrophic mutants. For example, using arginine auxotrophs of *C. glutamicum* and limiting the amount of L-arginine in the medium led to the release of the inhibition of L-arginine on N-acetylglutamate synthase and N-acetylglutamokinase, as well as the repression of other enzymes, and thus the overproduction of L-ornithine [6]. Similarly, the overproduction of L-lysine was observed in homoserine auxotrophs of *C. glutamicum* by limiting the concentration of L-homoserine (L-threonine plus L-methionine), thus releasing feedback inhibition on aspartokinase by L-threonine plus L-lysine [7]. L-Citrulline production by arginine auxotrophs of *Bacillus subtilis* [8] and *C. glutamicum* were also reported.

However, this application of auxotrophs is rather restricted, because the amino acids are supplied from the media and the negative feedback mechanism is not released.

3.3 Genetic Desensitization of Key Enzymes to Feedback Inhibition and Selection of Amino Acid Analog-Resistant Mutants

It has been shown that amino acid analog-resistant mutants of *E. coli* excrete amino acids into their surrounding media, such as L-methionine by ethionine-resistant strains [9], L-tryptophan by 5-methyltryptophan-resistant strains [10], L-threonine by α-amino-β-hydroxyvaleric acid (AHV)-resistant mutants [11], and others. Cohen and Patte also showed that homoserine dehydrogenase, a key enzyme of threonine biosynthesis, was insensitive to feedback inhibition by L-threonine in AHV-resistant mutants. These analog-resistant mutants excrete only a small amount of amino acids; the exact amount produced was not described in these reports, possibly due to the interest of these authors simply in their production

"ability" [11]. On the other hand, AHV-resistant mutants derived from *B. flavum* (later reclassified as *C. glutamicum*) produce a large amount of L-threonine [12]. The amount produced was large enough to be industrialized, and this was the first successful use of an analog-resistant mutant for large-scale amino acid production. Homoserine dehydrogenase of the mutant strain is 1,300-fold more insensitive to feedback inhibition by L-threonine than the parent strain [13]. The mutation that impairs feedback was found to be the replacement of one amino acid in the C-terminal region of the protein [14, 15].

Following this, various amino acid-producing strains were derived from their corresponding analog-resistant mutants: L-lysine, L-isoleucine, L-tryptophan, L-histidine, L-valine, L-phenylalanine, L-glutamine, L-arginine, L-proline, L-serine, and others from the progenitor strains of *B. flavum*, *B. lactofermentum*, *C. glutamicum*, *S. marcescens*, *E. coli*, and so on. Many of these mutants were shown to have key enzymes that were desensitized to feedback inhibition, and a number of these strains were successfully put into industrialization (as described in Sects. 4.2–4.9 of this chapter).

3.4 Disruption of Amino Acid Degradation Activity

Enteric bacteria, such as *E. coli* and *S. marcescens*, possess activities to degrade amino acids.

When using these bacteria as the parent strains for the breeding of amino acid producers, these activities prevented production. Therefore, it was necessary to abrogate these activities for use of these strains to produce amino acids. Mutants with disrupted degradation activities were derived from strains that could not assimilate amino acids as sources of carbon or nitrogen. However, amino acid overproduction has never been observed in strains containing only mutations that disrupt these processes.

3.5 Amplification of Genes Coding for Desensitized Biosynthetic Enzymes

Recombinant DNA techniques have made it possible to amplify and modify genes that code for biosynthetic enzymes. L-Threonine production by *E. coli* mutants was vastly improved by the amplification of desensitized L-threonine biosynthetic enzymes [16].

Thereafter, many more strains that have improved yields of various amino acids by the application of recombinant DNA techniques have been reported.

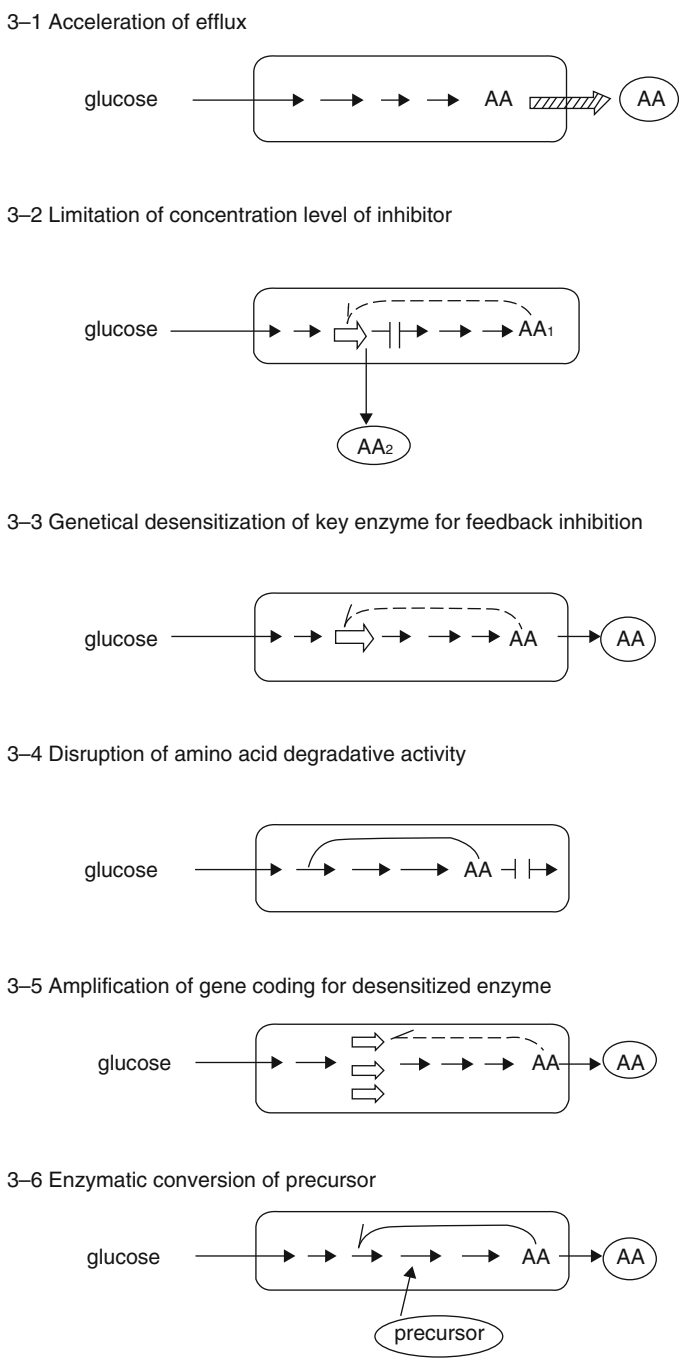

Fig. 3 Schematic illustration of key techniques for amino acid production. ⇒: acceleration of efflux, ⇒: release or desensitized enzyme, ⇒: amplification of genes coding for

3.6 Application of Metabolic Enzyme Reactions Free From Feedback Controls of the End Products

Ordinary L-aspartic acid biosynthesis via pyruvic acid is regulated by the levels of intracellular L-aspartic acid, but the reaction for the conversion of fumaric acid to L-aspartic acid is not regulated. Various microorganisms able to convert fumaric acid to L-aspartic acid by aspartase were selected, which allowed ammonium fumarate to be effectively converted to L-aspartic acid. The process was further improved using immobilized cells containing aspartase on either a polyacrylamide gel or a k-carrageenan gel [17].

Similarly, production of L-alanine from L-aspartic acid, L-tryptophan from indole plus serine or from anthranilic acid, L-serine from glycine, L-threonine from homoserine, and L-isoleucine from DL-α-aminobutyric acid has been reported. These enzyme reactions are all characterized by the absence of regulation by intracellular amino acid levels.

However, these reactions were not of practical use because the required precursors are too expensive. Only L-aspartic acid and L-alanine synthesis were put into practical use as described in Sect. 3.6.

Chemically synthesized intermediates of amino acids can be converted to their corresponding amino acids through the use of enzymes from microorganisms that are able to assimilate these synthesized intermediates as carbon or nitrogen sources. For these reactions, microorganisms that can convert hydantoin compounds and thiazoline compounds to their corresponding D- or L-amino acids were selected, including those that produced D-*p*-hydroxyphenylglycine and L-cysteine, which were industrialized by the Kaneka Co. and the Ajinomoto Co., respectively.

These key techniques (3-1 through 3-6) are illustrated schematically in Fig. 3.

4 Selection and Construction of Amino Acid-Producing Strains

4.1 L-Glutamic Acid

As described in detail in Sect. 3, L-glutamic acid fermentation was accomplished through the finding of the newly isolated strain *C. glutamicum*. This bacterium was isolated through a unique bioassay system that employed lactic acid bacteria, which require amino acids for growth. Naturally occurring samples of bacteria were screened on plates of minimal medium supplemented with lactic acid bacteria

Fig. 3 (continued) desensitized enzyme, ◄---------: release of feedback inhibition, —┤ ├►: block of reaction, (AA): amino acid produced

and all required amino acids except L-glutamic acid. Halo-forming colonies on plates, which showed excretion of L-glutamic acid, were then selected easily [18].

4.2 L-Lysine

Production of L-lysine was attained by culturing homoserine bradytrophs of *C. glutamicum* under limiting concentrations of homoserine (L-threonine plus L-methionine) in culture media, as described in Sect. 3.2 (Nakayama et al. 1958). Mutants of the homoserine auxotrophs of *B. flavum*, which show phenotypic sensitivity to threonine or methionine, are also high L-lysine producers. Sensitivity comes from repression by L-methionine or inhibition by L-threonine, respectively, which affects the homoserine dehydrogenase of the strain [19].

Resistant mutants of *B. flavum* to S-(2-aminoethyl)-L-cysteine (AEC), an analog of L-lysine, produce large amounts of lysine [20]. Aspartokinase, a key enzyme of lysine and threonine biosynthesis, was mutated to become insensitive to feedback inhibition by threonine plus lysine [20]. *B. lactofermentum* strains, also derived from mutants resistant to AEC as well as other analogs and an alanine auxotroph, produce a large quantity of L-lysine [21]. These days, genetically constructed *E. coli* strains with much higher yields are used in practical production.

4.3 L-Threonine

L-Threonine is both used for medicine and as a feed additive, which has recently been in high demand. As described in Sect. 3.3, strains that produce L-threonine were selected as AHV-resistant mutants of *B. flavum* [12], *C. glutamicum* [22], and *S. marcescens* [23].

Deriving an isoleucine and methionine double auxotroph and culturing in L-isoleucine- and L-methionine-limiting conditions improved L-threonine production by an AHV-resistant mutant of *E. coli*. Increased production can be explained by the release of repression by L-isoleucine plus L-threonine of the L-threonine biosynthetic enzymes and by methionine of the methionine-sensitive aspartokinase and homoserine dehydrogenase [24].

As described in Sect. 3.5, production of L-threonine was greatly improved by the amplification of genes coding for the desensitized L-threonine biosynthetic enzymes through the application of recombinant DNA techniques in *E. coli* [16]. The production was further improved and stabilized by integrating the genes on the bacterial chromosome using a defective Mu phage system [25]. This strain has been used in practical production of amino acids.

Improved production of L-threonine by the application of DNA techniques has also been reported for strains of *B. flavum*, *B. lactofermentum*, *C. glutamicum*, and *S. marcescens*; however, it is not certain whether these results were put into

practical use [26]. These days, genetically constructed *E. coli* strains with much higher yields are used in practical production.

4.4 L-Tryptophan

L-Tryptophan is a promising amino acid for use as a feed additive. Approaches for its production have a long history as follows: (a) enzymatic conversion of precursors, such as indole, pyruvate, and ammonia using tryptophanase, and indole plus L-serine using tryptophan synthase; (b) fermentative conversion of anthranilate; (c) enzymatic conversion of a DL-hydantoin compound, an intermediate for chemical synthesis of DL-tryptophan, to L-tryptophan; and (d) direct production from sugars. Method (d) is the most economical because precursors used in (a) through (c) are expensive. In the biosynthetic pathway of L-tryptophan, the main regulation sites are 3-deoxy-D-*arabino*heptulosonate-7-phosphate (DAHP) synthase and anthranilate synthase. Construction of strains capable of producing L-tryptophan was carried out through selection of mutants resistant to various tryptophan analogs.

A typical example of a producing strain was derived from *B. flavum*; this mutant is a tyrosine-auxotrophic, 5-fluorotryptophan-, and azaserine-resistant mutant [27]. Other mutants include a *C. glutamicum* tyrosine and phenylalanine double auxotrophic strain and several analog-resistant mutants (5-methyltryptophan, tryptophan hydroxamate, tyrosine hydroxamate, phenylalanine hydroxamate, 6-fluorotryptophan, 4-methyltryptophan, and *p*-fluorophenylalanine) [28] and mutants of *B. subtilis* resistant to 5-fluorotryptophan and indolmycin [29]. These strains are able to produce 10–15 g/l of L-tryptophan and are used in practical fermentations. These days, genetically constructed *E. coli* strains with much higher yields are used in practical production.

4.5 L-Phenylalanine

There is a large demand for L-phenylalanine as it is a component of aspartame, a low-calorie sweetener. The biosynthesis and feedback regulations of L-phenylalanine, L-tyrosine, and L-tryptophan were described in Sect. 4.4. DAHP synthase and the chorismate mutase-prephenate dehydratase complex are key enzymes in L-phenylalanine production. The best producing strain reported was derived from *B. lactofermentum* as a mutant resistant to *p*-fluorophenylalanine and 5-methyltryptophan while being sensitive to decoinine, an analog of purine; this strain is able to produce 25 g/l by the cultivation in a medium containing fumarate and acetate with 13% glucose [30]. These days, genetically constructed *E. coli* strains with much higher yields are used in practical production.

4.6 L-Proline

An industrialized L-proline-producing strain was derived from *B. flavum* as an isoleucine auxotrophic and sulfaguanidine- and dehydroproline-resistant mutant [31]. Another producing strain was also derived from *S. marcescens* in the form of a proline oxidase-deficient and proline analog-resistant mutant [32, 33]. Improvement of their yield was attained by the amplification of the *proAB*, the gene coding for L-proline biosynthetic enzyme from the *S. marcescens*-overproducing strain itself [32, 33].

4.7 L-Serine

L-Serine is produced by the fermentative conversion of glycine. A mutant with a low activity for L-serine degradation derived from *C. glycinophilum* can produce 14 g/l of L-serine from glycine with a molar yield of 33% [34]. Several other bacteria, such as those belonging to *Nocardia*, *Sarcina*, methanol-utilizing bacteria, *Pseudomonas*, and *Hyphomicrobium* sp., were also found to produce L-serine from glycine [35]. Production of L-serine from glucose without addition of glycine was also discovered using an azaserine-resistant mutant derived from an L-serine-nondegradative strain of *B. flavum* [36], which has been in use for practical production.

4.8 L-Histidine

L-Histidine-producing strains were selected from various analog-resistant mutants of *C. glutamicum*, *B. flavum*, and *S. marcescens*. The *C. glutamicum* strain, which was selected sequentially as a mutant resistant to 1,2,4-triazolealanine, 6-mercaptopurine, 8-azaguanine, 2-thiouracil, 6-methylpurine, and 5-methyltryptophan, can produce about 15 g/l of L-histidine. The *B. flavum* strain, which was selected as a mutant resistant to 2-thiazolealanine, sulfaguanidine, AHV, ethionine, and 2-aminobenzothiazole, produces 10–12 g/l of L-histidine. A *S. marcescens* mutant, which has low L-histidine-degradative activity, both through a feedback-insensitive enzyme and derepressed L-histidine biosynthetic enzymes combined with transductional techniques and 6-methylpurine resistance, can produce 23 g/l of L-histidine. Recombinant DNA techniques have been applied to amplify genes for the biosynthetic enzymes, and improvement of yields has been reported in the above-described bacteria [37].

4.9 L-Arginine, L-Ornithine, and L-Citrulline

L-Arginine-producing bacteria were derived from both arginine and other analog-resistant or sensitive mutants. An isoleucine-bradytrophic, D-serine-sensitive, D-arginine-resistant, arginine hydroxamate-resistant mutant of *C. glutamicum*, a sulfaguanidine- and 2-thiazolealanine-resistant mutant of *B. flavum*, and an arginine hydroxamate- and 6-azauracil-resistant mutant of *B. subtilis* can each produce 25–35 g/l of L-arginine. An improved producer was derived from *S. marcescens*, which has an arginine-nondegradative activity and feedback-insensitive and derepressed arginine biosynthetic enzymes introduced through transduction [38]. Production of L-ornithine and L-citrulline is described in Sect. 3.2.

4.10 L-Aspartic Acid

L-Aspartic acid is used both as a medicine and as a component of aspartame, a low-calorie sweetener composed of a dipeptide from aspartic acid and phenylalanine methyl ester. L-Aspartic acid was the first amino acid produced by enzymatic synthesis. As described in Sect. 3.6, this amino acid is formed in a reaction mixture containing *E. coli* cells from a strain that exhibited high activity of aspartase and ammonium fumarate [39]. A continuous production process was established with these *E. coli* cells immobilized on a polyacrylamide gel and then on a k-carrageenan gel [40]. Direct production from glucose using *B. flavum* mutants has been reported; however, their productivity was not so high.

4.11 L-Alanine

DL-Alanine is now practically produced through chemical synthesis from acetaldehyde, ammonia, and cyanide. Microbially produced alanine from glucose forms in the DL-form. An L-alanine-producing strain was constructed by deriving an alanine racemase-deficient mutant of *Arthrobacter oxydans*, which was selected to be a strain possessing a glucose-nonrepressible alanine dehydrogenase. Production of L-alanine was 75 g/l in a medium containing 14.5% glucose [41]; however, practical use of the strain is uncertain.

L-Alanine is produced from L-aspartate by aspartate-β-decarboxylase. *Ps. dacunhae* (now reidentified as *Comamonas testosteroni*) was found to be the best producer of this enzyme, and optimal conditions were determined [42]. The process was improved by use of immobilized cells of *Ps. dacunhae* in k-carrageenan gels. Further, the process for direct formation of L-alanine from fumarate, a substrate of aspartate, was developed by the combination of immobilized cells containing

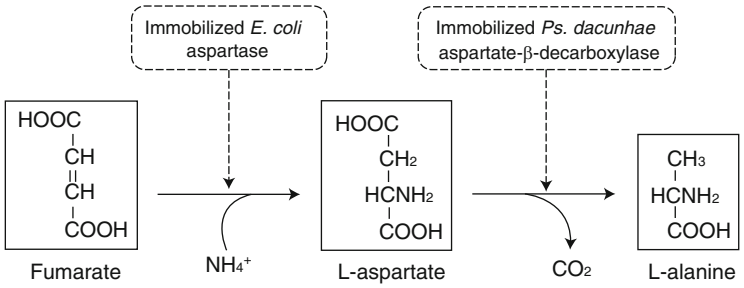

Fig. 4 Enzymatic synthesis of L-aspartic acid and L-alanine from fumaric acid with immobilized aspartase and aspartate-β-decarboxylase

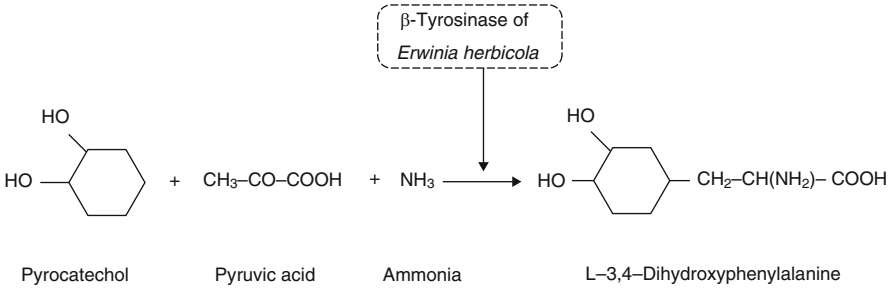

Fig. 5 Enzymatic synthesis of L-3,4-dihydroxyphenylalanine with β-tyrosinase of *Erwinia herbicola*

aspartase of *E. coli* and aspartate-β-decarboxylase of *Ps. dacunhae*, respectively, as shown in Fig. 4 [43].

4.12 L-3,4-Dihydroxyphenylalanine

L-3,4-Dihydroxyphenylalanine (Dopa) is not found in microbial sources but in brain organs and several plants. The compound is used for the treatment of Parkinson's disease. Enzymatic production of L-Dopa is achieved by the application of β-tyrosinase, which catalyzes the α,β-elimination of L-tyrosine to form pyruvic acid, phenol, and ammonia. Synthesis of L-Dopa is achieved by means of β-replacement between pyruvic acid, ammonia, and pyrocatechol using the enzyme from *Erwinia herbicola* (Fig. 5). A final concentration of 55 g of L-Dopa per liter of reaction mixture was obtained by the addition of a limited concentration of pyrocatechol [44, 45], and the process was industrialized by the Ajinomoto Co. in 1992.

4.13 L-Cysteine

L-Cysteine is now produced by extraction from acid hydrolysates of hair or feathers and enzymatic conversion of chemically synthesized DL-2-amino-Δ^2thiazole-4-carboxylic acid (ATC), an intermediate compound of DL-cysteine, to L-cysteine. Enzymes (ATC racemase, ATC hydrolase, and s-carbamoylcysteine hydrolase) from a newly isolated bacterium, *Ps. thiazolinophilum*, which can assimilate DL-ATC as a nitrogen source, can effectively form L-cysteine from DL-ATC [46], a process that was put into practical production in 1980 by the Ajinomoto Co. Now cysteine is also produced industrially by fermentation.

4.14 D-p-Hydroxyphenylglycine

D-Hydantoinase catalyzes the hydrolysis of 5-substituted hydantoin derivatives, which are synthesized chemically as precursors of amino acids to form N-carbamoyl-D-amino acids. The enzyme is distributed widely in microorganisms, and various N-carbamoyl-D-amino acids can be obtained by the enzymes from corresponding 5-substituted hydantoin compounds. Among them, production of D-*p*-hydroxyphenylglycine, a building block for a semisynthetic β-lactam antibiotic, amoxicillin, from DL-*p*-hydroxyphenylhydantoin by using the enzyme of *Ps. putida* followed by chemical hydrolysis of the intermediate N-carbamoyl-D-*p*-hydroxyphenylglycine to D-*p*-Hydroxyphenylglycine, was established. Thereafter, the chemical process, which had several weak points, including use of large amounts of acids and alkalis and the appearance of by-products, was improved by replacement with a biochemical one, as follows: (a) selection of a strain belonging to *Agrobacterium* sp. that had high D-carbamylase activity, (b) modification of the gene to form a heat-stable carbamylase using an established *E. coli* system, (c) large-scale production by employing an *E. coli* strain, and (d) immobilization of the enzyme on an ion-exchange resin, such as Duolite A568. Combining this system with that of immobilized hydantoinase, a bioreactor system (Fig. 6) for the

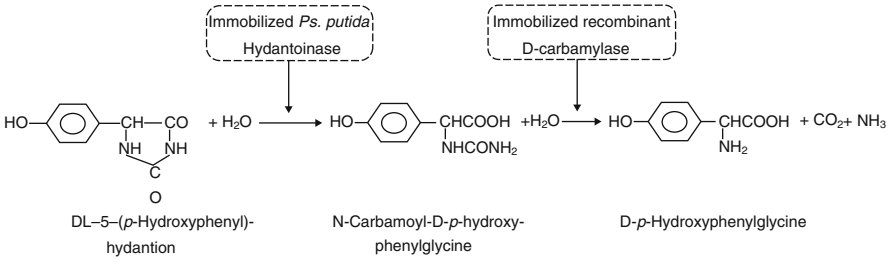

Fig. 6 Enzymatic synthesis of D-*p*-hydroxyphenylglycine from DL-5-(*p*-hydroxyphenyl)-hydantoin with the combination of hydantoinase and D-carbamylase

production of D-*p*-hydroxyphenylglycine, has been industrialized by the Kaneka Co. [47, 48].

4.15 L-4-Hydroxyproline

Hydroxyproline theoretically has eight isomers, among which the naturally occurring *trans*-4-hydroxyl-L-proline is a component of collagen and sugar proteins. This amino acid is used as a starting material for various pharmaceuticals, such as optically active proline and pyrrolidine derivatives. The production of this substance had been carried out through hydrolysis of collagen. The recently developed microbial production process included (a) screening of microbial proline hydrooxigenizing enzymes from strains belonging to *Streptomyces* and *Dactylosporangium*, (b) cloning and amplification of the gene coding for this enzyme using a gene expression system in *E. coli*, and (c) construction of a hydroxyproline-producing strain of *E. coli* using this expression system as well as incorporating a proline-nondegradative activity and L-proline-overproducing ability [49]. The process was industrialized by the Kyowa Hakko Co. in 2000.

5 Discussion and Future Prospects

In view of the early history of amino acid production, research and development as well as modification into practical production has been carried out mainly by Japanese companies, who hold a leading position in this field in the world today though some of the companies have changed the policy.

Demand for amino acids will increase as the population of the world continues to increase, and amino acids cannot be replaced by any other substances; further, novel uses for amino acids will also be developed. Amino acids used for feed additives, which include L-lysine, L-threonine, L-tryptophan, and chemically synthesized DL-methionine, compete with natural sources, such as soybean meal and fish meal. Prices of these materials vary according to their supply. Thus, a stable and cheap supply of amino acids is important. Judging from the numbers of publications, recent research and development activities appear to have decreased when compared with those of the twentieth century. Further efforts for improvement in the construction of strains, search for raw materials, cultivation, separation and purification, and reduction of environmental load are still required.

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Part II
Examples of Amino Acids Fermentation

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Glutamate Fermentation-2: Mechanism of L-Glutamate Overproduction in *Corynebacterium glutamicum*

Takashi Hirasawa and Masaaki Wachi

Abstract The nonpathogenic coryneform bacterium, *Corynebacterium glutamicum*, was isolated as an L-glutamate-overproducing microorganism by Japanese researchers and is currently utilized in various amino acid fermentation processes. L-Glutamate production by *C. glutamicum* is induced by limitation of biotin and addition of fatty acid ester surfactants and β-lactam antibiotics. These treatments affect the cell surface structures of *C. glutamicum*. After the discovery of *C. glutamicum*, many researchers have investigated the underlying mechanism of L-glutamate overproduction with respect to the cell surface structures of this organism. Furthermore, metabolic regulation during L-glutamate overproduction by *C. glutamicum*, particularly, the relationship between central carbon metabolism and L-glutamate biosynthesis, has been investigated. Recently, the role of a mechanosensitive channel protein in L-glutamate overproduction has been reported. In this chapter, mechanisms of L-glutamate overproduction by *C. glutamicum* have been reviewed.

Keywords 2-Oxoglutarate dehydrogenase complex, *Corynebacterium glutamicum*, DtsR, L-Glutamate, LtsA, Mechanosensitive channel, NCgl1221, OdhI

Contents

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1 Introduction

As reviewed in the previous chapters, the discovery of *Corynebacterium glutamicum* has enabled us to produce L-glutamate, which is known as a flavor enhancer, by microbial fermentation. This microorganism has also been utilized for industrial fermentative production of various amino acids such as L-lysine and L-valine.

The amount of L-glutamate produced by *C. glutamicum* is notably high and the wild-type *C. glutamicum* strain does not produce any amino acids other than L-glutamate. Therefore, it was thought by many researchers that the metabolic pathways related to L-glutamate biosynthesis were different from those of the other microorganisms. However, later on, it was found that the L-glutamate biosynthetic pathway in *C. glutamicum* was identical to those of other microorganisms.

C. glutamicum undergoes unique cell division called snapping division; it grows as V-shaped cell groups after the septation followed by cell division (Fig. 1). In addition, *C. glutamicum* has distinct cell wall structures; the peptidoglycan layer is covered with an additional outer layer, mainly composed of mycolic acids, which are long-chain fatty acids, R_1-CH(OH)-CH(R_2)-COOH (R_1 and R_2 are the alkyl chains). The existence of this mycolic acid-containing layer in the cell wall may be somehow involved in the mechanism of snapping division in *C. glutamicum*.

L-Glutamate fermentation by *C. glutamicum* can be induced by limitation of biotin, which is a necessary compound for its growth [1]. It can also be induced by addition of fatty acid ester surfactants such as Tween 40 [2], addition of β-lactam antibiotics such as penicillin G [3], or addition of inhibitors that affect formation of cell surface layer containing mycolic acids such as ethambutol [4]. It had been speculated that the produced L-glutamate passively leaks through cell surface structures of *C. glutamicum* because the treatments to induce L-glutamate production were thought to affect cell surface structures of this bacterium. Therefore,

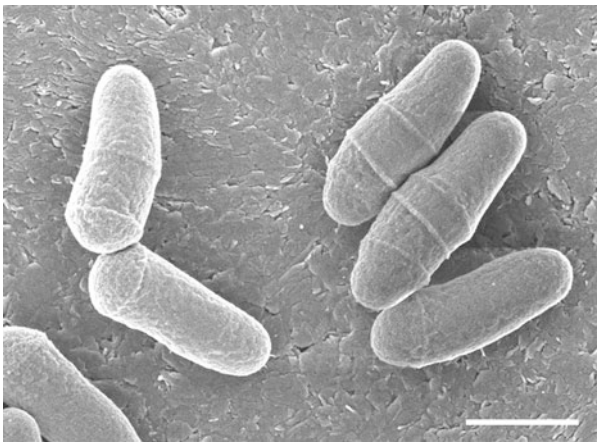

Fig. 1 Scanning electron microphotograph of *C. glutamicum* cells. Scale bar: 1 μm

many researchers had investigated the changes in cell surface components during L-glutamate production by *C. glutamicum*. Subsequently, regulation of the central carbon metabolism during L-glutamate production, based on the decrease in 2-oxoglutarate dehydrogenase complex (ODHC) activity, was reported. In addition, the protein responsible for this metabolic regulation, OdhI, was identified. Further, the contribution of mechanosensitive channel protein NCgl1221 in the excretion of L-glutamate by *C. glutamicum* was reported.

In this chapter, analyses of the mechanism involved in L-glutamate overproduction by *C. glutamicum* have been reviewed with emphasis on metabolic regulation, relationship between cell surface structures and L-glutamate production, and the role of mechanosensitive channel.

2 Metabolic Regulation in L-Glutamate Production by *C. glutamicum*

Since the amount of L-glutamate produced by *C. glutamicum* is significantly high, it was believed that *C. glutamicum* possesses unique biosynthetic pathways for L-glutamate biosynthesis. As shown in Fig. 2, however, the cellular metabolisms related to L-glutamate biosynthesis are same as those in other microorganisms; L-glutamate is synthesized from 2-oxoglutarate and ammonia, and its biosynthetic reaction is catalyzed by glutamate dehydrogenase (GDH), with NADPH as a reducing equivalent [5]. In addition, 2-oxoglutarate is synthesized from isocitrate and converted further to succinyl-CoA in the TCA cycle. The conversion of 2-oxoglutarate to succinyl-CoA is catalyzed by ODHC, consisting of a catalytic subunit E1o (OdhA; NCgl1084), dihydrolipoamide acetyltransferase subunit E2 (AceF; NCgl2126), and dihydrolipoamide dehydrogenase subunit E3 (Lpd;

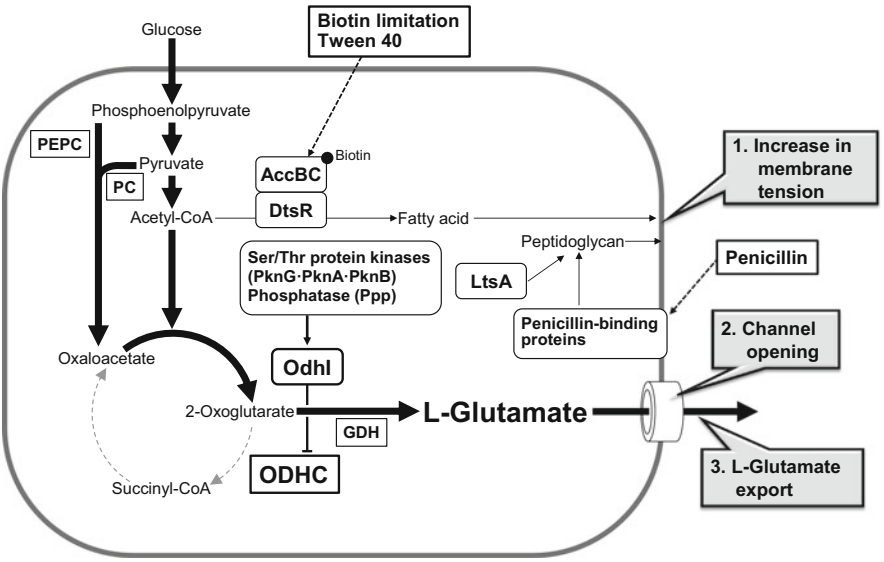

Fig. 2 Overview of L-glutamate production mechanism in *C. glutamicum*

NCgl0355). The regulatory mechanisms in the L-glutamate metabolism of *C. glutamicum* have been investigated extensively.

2.1 *The Decrease in ODHC Activity During L-Glutamate Production by* C. glutamicum

Initially, while investigating the mechanism of L-glutamate production, the activity of ODHC could not be detected in *C. glutamicum*. It was therefore believed by many researchers that this is the reason for outstanding productivity of L-glutamate by this bacterium. However, Kawahara et al. succeeded to detect the activity of ODHC and found that the ODHC activity was decreased during L-glutamate production induced by the biotin limitation, Tween 40 addition, and penicillin addition [6]. Since ODHC is located at the branching point between the TCA cycle and L-glutamate biosynthesis, it was widely assumed that a change in metabolic flow from the TCA cycle to L-glutamate synthesis caused by a decrease in ODHC activity induces L-glutamate production.

Afterward, Shimizu et al. investigated the metabolic impact of the change in activity of enzymes related to L-glutamate biosynthesis, isocitrate dehydrogenase (ICDH), which catalyzes the formation of 2-oxoglutarate [7], ODHC and GDH, by metabolic control analysis [8, 9]. As expected, the change in the activities of ICDH and GDH had a small impact only on L-glutamate production, but the change in the activity of ODHC had a great impact on the L-glutamate production induced by

biotin limitation. Metabolic flux analysis during L-glutamate production also supported this phenomenon. In addition, the Michaelis–Menten constant of ODHC for 2-oxoglutarate was lower than that of GDH, indicating that the affinity of ODHC to 2-oxoglutarate was higher than that of GDH. These results suggest that the accumulation of 2-oxoglutarate due to the decreased ODHC activity is required for the increased metabolic flux of L-glutamate production.

2.2 Discovery of the OdhI Protein and Its Role in L-Glutamate Production

In 2006, Niebisch et al. reported the discovery of a novel protein, OdhI, in *C. glutamicum* [10]. They found that the mutant strain for *pknG* gene, encoding a serine/threonine protein kinase, showed a defect in growth on glutamine. OdhI (NCgl1385) was identified as one of the target proteins of PknG, based on proteomic analysis. The OdhI protein has a fork-head-associated (FHA) domain, which is known to be responsible for its interaction with other proteins. The PknG protein phosphorylates Thr14 and/or Thr15 residues of the OdhI protein. Moreover, unphosphorylated OdhI protein interacts with OdhA protein (E1o subunit) of ODHC via FHA domain and, thereby, inhibits ODHC activity. Since ODHC is essential for the growth on glutamine, the *pknG* mutant showed growth defect on glutamine due to the inhibition of ODHC activity by unphosphorylated OdhI. The crystal structure of OdhI protein suggests an autoregulatory mechanism; the N-terminal region of the phosphorylated OdhI protein masks the FHA domain itself, and as a result, it cannot interact with the OdhA protein [11].

Schultz et al. examined L-glutamate production by the *odhI* mutant of *C. glutamicum* under biotin limitation, Tween 40 addition, penicillin addition, and ethambutol addition; ethambutol is known as an inhibitor for mycolic acid layer formation in the cell wall structures [12]. The *odhI* mutant strain produced almost zero or very small amounts of L-glutamate under these induction treatments, suggesting that the L-glutamate production by *C. glutamicum* is dependent on OdhI.

Kim et al. reported that penicillin-induced L-glutamate production was inhibited by chloramphenicol, a de novo protein synthesis inhibitor, indicating that the de novo protein synthesis is required for L-glutamate production in *C. glutamicum* [13, 14]. Therefore, they performed the proteomic analysis of *C. glutamicum* during penicillin-induced L-glutamate production to identify the protein(s) required for L-glutamate production by *C. glutamicum*. OdhI was one of the proteins upregulated by addition of penicillin. These results suggested that the de novo synthesis of OdhI protein is required for penicillin-induced L-glutamate production by *C. glutamicum*.

Kim et al. also investigated the phosphorylation status of OdhI during L-glutamate production induced by Tween 40 and penicillin additions [13, 15]. Under both L-glutamate production conditions, the amount of unphosphorylated OdhI was high. Boulahya et al. also observed the dephosphorylation of OdhI during biotin-limited

and the temperature-induced L-glutamate production [16]. However, no clear relation between the specific L-glutamate production rate and OdhI phosphorylation level has been demonstrated, suggesting that the phosphorylation status of OdhI might not be the only factor necessary for the induction of L-glutamate production in *C. glutamicum*.

2.3 *Contribution of Anaplerotic Reactions to L-Glutamate Production by* C. glutamicum

In the anabolic metabolism, anaplerotic reactions are important to supply oxaloacetate in the TCA cycle, because the intermediate metabolites of the TCA cycle are utilized for biosynthesis of amino acids and fatty acids. *C. glutamicum* carries two anaplerotic reactions catalyzed by phosphoenolpyruvate carboxylase (PEPC) [17] and pyruvate carboxylase (PC) [18]. Since the anaplerotic reactions are essential for L-glutamate biosynthesis, the contribution of anaplerotic reactions to L-glutamate production by *C. glutamicum* was analyzed.

We investigated the redistribution of metabolic flux in *C. glutamicum* during L-glutamate production induced by biotin limitation [19]. Under the biotin limitation, it is thought that PC cannot function because biotin is a cofactor of PC. Indeed, the mutant strain for PEPC could not produce L-glutamate under biotin limitation, because both anaplerotic reactions did not function in this mutant. Overexpression of *ppc* encoding PEPC increased L-glutamate production. Metabolic flux analysis of *C. glutamicum* during L-glutamate production was performed; cells were grown on ^{13}C-labeled glucose under biotin limitation and information on ^{13}C-labeling of L-glutamate produced was obtained by nuclear magnetic resonance spectroscopy. The metabolic flux for anaplerotic reactions in the mutant for PC was lower than that in the wild-type strain. These results suggest that the anaplerotic reaction catalyzed by PEPC contributes to L-glutamate production under biotin limitation.

Shirai et al. investigated the metabolic flux redistribution in *C. glutamicum* ATCC 13869 strain during L-glutamate production induced by Tween 40 addition, using ^{13}C-metabolic flux analysis [13, 20]. ^{13}C-metabolic flux analysis is recognized as a powerful tool to determine the precise metabolic flux distributions and for evaluating cellular metabolic state [21, 22]. Cells were grown on ^{13}C-labeled glucose, and biomass hydrolysate was obtained after the addition of Tween 40 to induce L-glutamate production. Then, the ^{13}C-labeling information of the amino acids in the cellular proteins was obtained using gas chromatography–mass spectrometry. The metabolic flux distributions were determined, taking into consideration the glycolysis, the pentose phosphate pathway, the TCA cycle, and the anaplerotic reactions. Under the Tween 40 addition, the flux for PC was enhanced, while not for PEPC. This suggests that the anaplerotic reaction catalyzed by PC contributes to L-glutamate production induced by Tween 40 addition.

3 Relationship Between Cell Surface Structure and L-Glutamate Production in *C. glutamicum*

As described above, the L-glutamate production by *C. glutamicum* is induced by biotin limitation, Tween 40 addition, and penicillin addition. Since these treatments have been thought to affect cell surface structures of *C. glutamicum*, some researchers investigated the relationship between the cell surface, i.e., cell membrane and cell wall, and L-glutamate production.

3.1 Relationship Between the Fatty Acid Content and L-Glutamate Production in C. glutamicum

Hoischen and Krämer investigated the relationship between the content and composition of lipids in the cytoplasmic membrane and L-glutamate secretion [23]. They found that the change in fatty acid or phospholipid contents and their composition were not apparently related to L-glutamate secretion, although the overall lipid content was reduced. These suggest that alteration of lipid content and/or its composition in cytoplasmic membrane are not sufficient for induction of glutamate production.

Nampoothiri et al. investigated the effect of change in gene expression related to lipid synthesis, which leads to the modification of lipid contents in the membrane, on L-glutamate secretion ability of *C. glutamicum* [24]. Overexpression of *plsC* encoding acylglycerol acyltransferase increased L-glutamate production induced by Tween 60 addition, while overexpression of *acp* encoding acyl carrier protein decreased L-glutamate production.

Furthermore, Hashimoto et al. examined the change in mycolic acid composition in cell wall during L-glutamate production by *C. glutamicum* [25]. They found that the major mycolic acids in *C. glutamicum* are $C_{30–34}$ mycolic acids. The total amount of mycolic acids in *C. glutamicum* was decreased by induction treatments for L-glutamate overproduction. In case of biotin limitation and Tween 40 addition conditions, these treatments affect mycolic acid biosynthesis directly. On the other hand, in case of penicillin addition, the decrease in mycolic acid amount seems to result from a decrease in the peptidoglycan synthesis, because peptidoglycan layer functions as a scaffold for the outer layer, including the mycolic acid-containing layer in *C. glutamicum*. Moreover, under the biotin limitation condition, the content of short-chain mycolic acids was increased. Such alteration of the total amount and compositions of mycolic acids can be associated to L-glutamate overproduction by *C. glutamicum*. It is known that the mycolic acid-containing layer functions as a permeability barrier for extracellular compounds and is involved in maintaining the intracellular osmotic pressure. As described below, such alteration of mycolic acid-containing layer in *C. glutamicum* may be related to the function of mechanosensitive channel during L-glutamate overproduction.

3.2 *Role of DtsR Protein in Fatty Acid Biosynthesis and* L*-Glutamate Production in* C. glutamicum

The *dtsR* gene (NCgl0678) was identified as a multi-copy suppressor gene of a *C. glutamicum* mutant, which showed hypersensitivity to Tween 40 (palmitic acid ester surfactant) [26]. The amino acid sequence of the *dtsR* gene product showed strong homology with β-subunit of propionyl-CoA carboxylase of *Mycobacterium leprae* and β-subunit of acetyl-CoA carboxylase (AccC and AccD) of *Escherichia coli*, which are responsible for fatty acid biosynthesis. Moreover, a *dtsR*-disrupted mutant showed the auxotrophy of oleic acid [27]. The *dtsR* gene product would be responsible for the fatty acid biosynthesis.

The *dtsR*-disrupted mutant produced L-glutamate in the presence of excess biotin [28]. Overexpression of *dtsR* gene suppressed L-glutamate production induced by Tween 40 addition. Moreover, under biotin limitation and Tween 40 addition conditions, the levels of DtsR protein were decreased. These results suggest that change in the levels of DtsR protein is somehow related to the change in the ODHC activity.

3.3 *Function of LtsA Protein in Cell Surface Formation and* L*-Glutamate Production in* C. glutamicum

C. glutamicum shows high tolerance to a lytic enzyme, lysozyme, which catalyzes the hydrolysis of β-1,4 bonds between *N*-acetylglucosamine and *N*-acetylmuramic acid in the peptidoglycan, although it belongs to the Gram-positive bacteria. This is in turn facilitated by the unique mycolic acid-containing cell wall structure, which functions as a permeability barrier against the compounds outside *C. glutamicum* cells. We investigated the lysozyme-sensitive mutant strains of *C. glutamicum* for understanding the relationship between the cell surface formation and L-glutamate production [29, 30].

A mutant strain of *C. glutamicum* KY9714 showed high sensitivity to lysozyme, and temperature-sensitive growth at 37°C. Morphology of the KY9714 mutant was swollen at the restrictive temperature, which is one of the typical morphology of *C. glutamicum* temperature-sensitive mutants [31]. Therefore, it was thought that this mutant had a defect(s) in the cell surface formation. It was observed that the *ltsA* gene (NCgl2116) complemented the temperature-sensitive growth and lysozyme sensitivity of the KY9714 mutant. A nonsense mutation was found in the *ltsA* gene of the KY9714 mutant, and the *ltsA*-disrupted strain showed both temperature-sensitive growth and lysozyme sensitivity.

The amino acid sequence of LtsA protein shows high homology with those of glutamine-dependent asparagine synthetases, which belong to the *purF*-type glutamine-dependent amidotransferases, from various organisms. However, the *ltsA* gene could not complement the asparagine auxotrophy of *E. coli* asparagine

auxotrophic mutant (i.e., *asnA asnB* double mutant). This result suggested that the *ltsA* gene does not encode asparagine synthetase but glutamine-dependent amidotransferase responsible for the synthesis of cell wall component(s). Recently, Levefaudes et al. reported that the *C. glutamicum* LtsA protein is involved in amidation of *meso*-diaminopimelate (mDAP) residue in the peptidoglycan structure. The LtsA protein specifically amidates mDAP in the lipid intermediates during the peptidoglycan biosynthesis [32]. For understanding the detailed function(s) of the LtsA protein, further biochemical and molecular biological studies would be required.

The *ltsA* mutant strains produced L-glutamate on increasing the culture temperatures [29, 30]. At the restrictive temperatures, the cell surface rigidity is reduced in the *ltsA* mutant strains due to a defect of peptidoglycan amidation, which may induce the L-glutamate production.

4 A Role of the Mechanosensitive Channel Protein in L-Glutamate Secretion in *C. glutamicum*

In the last few decades of the twentieth century, Krämer and co-workers demonstrated that the excretion of L-glutamate, L-lysine, L-isoleucine, and L-threonine from *C. glutamicum* cells is an active process (for a review, [33]). Based on these pioneering studies, Eggeling and co-workers successfully identified novel transporters responsible for amino acid efflux, including LysE, TherE, and BrnFE, which export L-lysine, L-threonine, and L-isoleucine, respectively (for reviews, [34–36]) (also see Chap. 32). On the other hand, the nature of the L-glutamate exporter has long been unclear, although it was suggested that the secretion of L-glutamate by *C. glutamicum* is mediated by a carrier system in the cytoplasmic membrane [23, 37]. Recently, the contribution of a mechanosensitive channel protein on the cytoplasmic membrane in export of L-glutamate overproduced by *C. glutamicum* has been proposed.

4.1 Discovery of a Mechanosensitive Channel Protein NCgl1221

As described above, a wild-type *C. glutamicum* does not produce L-glutamate at all when cells grow happily. Cells produce large amounts of L-glutamate only under appropriate culture conditions. L-glutamate production is induced under biotin-limiting conditions, whereas the presence of biotin, which is required for *C. glutamicum* growth, inhibits its production [1]. L-Glutamate production is also induced by treatment with fatty acid ester surfactants [2] or penicillin [3]. Since biotin limitation and the other inducting treatments cause damage to the cell surface

structures of this microorganism, it has long been assumed that L-glutamate leaks through the cell membrane [38].

Moreover, the activity of ODHC decreases during L-glutamate production in response to inducing treatments [6]. It was reported that mutants of *odhA* gene, encoding a subunit of ODHC, produced L-glutamate constitutively [39]. During the analysis of such *odhA* mutants, we found that specific mutations in the NCgl1221 gene, but not *odhA* mutations, were responsible for constitutive L-glutamate secretion [40]. The NCgl1221 gene encodes a homolog of the small-conductance mechanosensitive channel (MscS). Disruption of NCgl1221 essentially abolishes L-glutamate secretion. Based on these findings, we proposed a hypothetical mechanism of L-glutamate production by *C. glutamicum* (Fig. 2). Inducible treatment causes an alternation in membrane tension, and the NCgl1221 mechanosensitive channel is then activated, which leads to L-glutamate excretion [40, 41].

Electrophysiological studies done by different research groups indicated that NCgl1221 indeed possesses mechanosensitive channel activity. Using *E. coli* giant spheroplasts, lacking MscS and MscL, it was shown that NCgl1221 exhibits the typical pressure-dependent gating behavior of a stretch-activated channel on patch-clump analysis [42]. The mechanosensitive channel activity of NCgl1221 was also confirmed by patch-clump analysis using giant provacuoles of *Bacillus subtilis* lacking mechanosensitive channels, MscL and YkuT (an MscS homolog) [43]. It was also shown that L-glutamate passes across the cytoplasmic membrane through the NCgl1221 mechanosensitive channel by passive diffusion [44]. These results indicate that NCgl1221 is a long-elusive L-glutamate exporter.

4.2 Functional Characterization of the Mechanosensitive Channel NCgl1221 in L-Glutamate Secretion in C. glutamicum

The NCgl1221 protein has an N-terminal domain (1–286 a. a.) homologous to the *E. coli* MscS and a long C-terminal domain (287–533 a. a.) of unknown function (Fig. 3a). In order to investigate the role of the C-terminal domain in L-glutamate secretion, a series of C-terminally truncated mutants of NCgl1221 were constructed and their ability for L-glutamate secretion was examined [45, 46] (Fig. 3b). It was shown that the N-terminal domain, homologous to *E. coli* MscS, retained the ability to cause L-glutamate secretion in response to the inducing treatments. In addition, it was also shown that a mutant with NCgl1221 lacking the C-terminal extracytoplasmic domain produced L-glutamate without any inducing treatments. These results suggest that the N-terminal domain is necessary and sufficient for the excretion of L-glutamate in response to inducing treatment and that the C-terminal extracytoplasmic domain has a negative regulatory role in L-glutamate production. Since the N-terminal domain alone can secrete L-glutamate in response to inducing

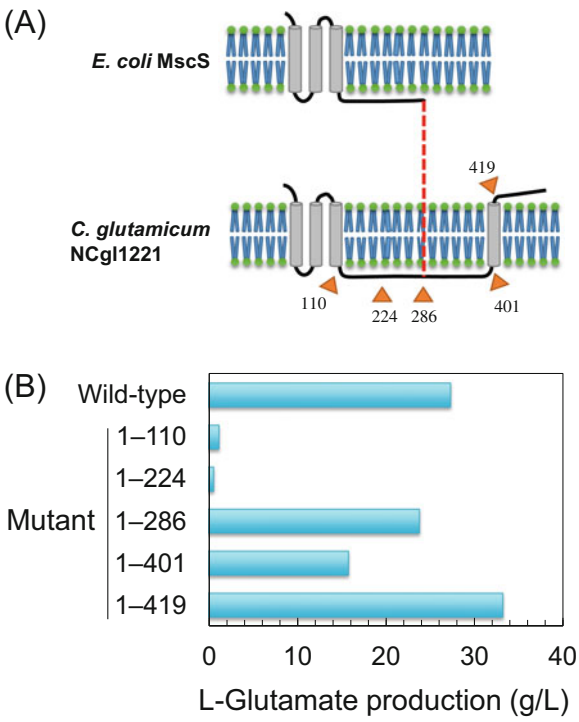

Fig. 3 Contribution of the mechanosensitive channel NCgl1221 to L-glutamate secretion in *C. glutamicum*. (**a**) Comparison of membrane topologies between *E. coli* MscS and *C. glutamicum* NCgl1221. *Arrowheads* indicate the positions of truncation for construction of mutant NCgl1221 proteins. (**b**) L-Glutamate production by the *C. glutamicum* harboring mutant NCgl1221 proteins. L-Glutamate production in the *C. glutamicum* wild-type strain and the NCgl1221-disrupted strain expressing NCgl1221 truncated at the *X*-th residues (1–*X*) under biotin limitation are shown. In case of the mutant 1–419, L-glutamate production under biotin-sufficient condition is shown

treatments, the precise role of the C-terminal domain, including the extracytoplasmic domain, remains obscure.

As mentioned above (see Sect. 4.2), the inducing treatments cause a decrease in ODHC activity, which change the metabolic flux from TCA cycle to biosynthesis of L-glutamate [6]. This metabolic regulation is conducted by the OdhI, an inhibitory protein of ODHC [10, 12]. Since a disrupted mutant of the *odhI* gene produces almost zero or very small amounts of L-glutamate under inducing conditions [12], the metabolic regulation by OdhI is definitively required for L-glutamate production by this bacterium. Overproduction of OdhI did induce L-glutamate production, but the production levels were much lower than observed under normal induction conditions [14]. On the other hand, gain-of-function mutants of NCgl1221 produce a large amount of L-glutamate constitutively without any inducing treatments [40, 47]. This means that the activation of NCgl1221 mechanosensitive channel is a prerequisite for L-glutamate production prior to a change in metabolic flux facilitated by OdhI. The relationship between NCgl1221 and OdhI is still to be elucidated.

C. glutamicum possesses only one MscL homolog and one MscS homolog as a mechanosensitive channel. A mutant lacking both channels, however, does not show increased sensitivity to hypo-osmotic shock [48]. Recent electrophysiological studies showed that the opening rates at saturating tensions and the closing rates at zero tension of NCgl1221 are at least one order of magnitude slower than those

observed for *E. coli* MscS. Moreover, whereas MscS is inactivated under sustained stimulus, NCgl1221 does not undergo inactivation. It is considered that the fast activation kinetics of MscS is advantageous for responding to an abrupt tension change upon an osmotic downshock. On the other hand, it seems that NCgl1221 is not suitable for the response to such fast changes, but is turned to execute slower processes, such as L-glutamate export [49]. That is, NCgl1221 mechanosensitive channel does not function as a safety valve for osmotic downshock, but should have another physiological function that remains to be elucidated.

Amino acid exporters are increasingly attracting attention for improving the industrial producer strains of amino acids (for reviews, [36, 50–52]). Overproduction of LysE and ThrE enhances the excretion of L-lysine and L-threonine, respectively, in their producer strains at least at laboratory scales [53, 54]. The NCgl1221 mechanosensitive channel shows a relatively lower specificity for substrates, although it exhibits a slight preference for cations over anions. Patch-clamp experiments showed that aspartic acid as well as potassium ion and chloride ion, in addition to L-glutamate, could pass thought this channel [42, 44, 49]. Introduction of gain-of-function mutant NCgl1221 into an *E. coli* proline producer strain improved its productivity [44]. These suggest a great potential of this channel for application in production of valuable molecules other than glutamate.

5 Conclusion and Future Perspectives

As described in this chapter, the molecular mechanisms of L-glutamate overproduction by *C. glutamicum* have become much clearer for this decade. However, the physiological role of L-glutamate overproduction, i.e., the reason why *C. glutamicum* produces and secretes significant amounts of L-glutamate, has not been understood. It is now expected that the L-glutamate production mechanisms can be applied to the production systems for useful chemicals.

The complete genomic DNA sequence of *C. glutamicum* has been already determined [55–59]. Therefore, genome-wide analysis such as transcriptomics, proteomics, metabolomics, and fluxomics for *C. glutamicum* are now available. For example, Kataoka et al. investigated the transcriptome of *C. glutamicum* during L-glutamate overproduction [60]. Moreover, systems biology studies, based on metabolic simulations, using genome-scale metabolic models have been carried out [61, 62]. Further studies on L-glutamate production mechanisms using such genome-wide analysis systems are required to understand the physiological roles of L-glutamate overproduction and to generate producer strains of useful chemicals, other than amino acids based on metabolic engineering.

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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.

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Contents

1 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 [1]. The global lysine market is currently estimated to be 2.2 million metric tons per year [2, 3] and is still growing at annual rates of around 10% (Fig. 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 [2, 3], depending largely on competition from natural lysine sources such as soybean meal and sardine (Fig. 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

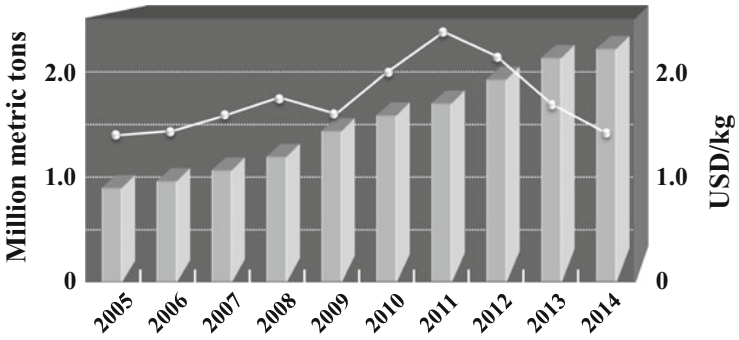

Fig. 1 Changes in estimated global markets (*bars*) and prices (*circles*) for lysine during the past decade

(Germany), among others [2, 3]. 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 [4]. 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 [5, 6]. 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 [7, 8]. 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 [9, 10]. 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 [11–14]. 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 [15–17]. 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 [3, 9], 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 [18–20].

This chapter first briefly describes the general outlines of lysine fermentation and typical production strains and 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 [9, 10, 14].

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 [21]. 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 (semicontinuous 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 [22].

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 [7]. 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 [7].

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 [9]. 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 coproducts 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 [23]. 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: (1) homoserine auxotrophy due to a defect of homoserine dehydrogenase and (2) AEC resistance due to the desensitization of aspartokinase from feedback inhibition [10, 24–26]. 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 [27, 28].

In addition to these fundamental properties, leucine auxotrophy is known to increase lysine production [29–31]. 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 [31]. More recently, however, since no significant upregulation 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 [32]. 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 [32].

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 [7, 9]. Additional screening and selection for these phenotypic characters has resulted in lysine production with yields of 40–50% on sugar [33].

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 [34–36]. Defect or reduction of phosphoenolpyruvate carboxykinase [37, 38], isocitrate dehydrogenase [39], or citrate synthase [40, 41] 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 [19]. 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 [42, 43], deletion of lysine-degradation pathways [44], amplification of the lysine exporter YbjE [45, 46], enhancement of respiratory chain pathways with high energy efficiency or reduction of those with low energy efficiency [47], disruption of the ribosome modulation factor [48], and overexpression of ppGpp synthetase [49]. 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 [20]. *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 [19]. *C. efficiens*, formerly named *Corynebacterium thermoaminogenes*, is phylogenetically close to *C. glutamicum* but can grow at temperatures approximately 10°C higher [50–52]. *B. methanolicus* is a thermotolerant methylotroph that grows at 35–60°C on methanol, an alternative carbon source which does not compete with human food [53].

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

Table 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	–	[4]
Brevibacterium flavum FA-3-115	AEC^{r}	Glucose 10% 30°C, 72 h	31.8	31.8	–	[54]
B. flavum No. 22	AEC^{r}, CS^{D}, PK^{D}, HD^{D}, and FP^{s}	Glucose 10% 30°C	51	51	–	[34]
C. glutamicum B-6	AEC^{r}, Rif^{r}, SM^{r}, and AU^{r}	Molasses 32°C, 48 h	100	–	2.1	[22]
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	–	[55]
C. glutamicum	Hse^{-}, Leu^{-}, and AEC^{r}	Glucose 18% 27°C, 70 h	60	33.3	–	[56]
C. glutamicum MH20-22B/pJC23	AEC^{r} (AK^{IS}) and Leu^{-} *dapA* on plasmid	Glucose 10%	50	50	–	[57]
Brevibacterium lactofermentum AJ12592	Hse^{-}, AEC^{r}, CCL^{r}, and DOL^{r}	Glucose 3.6% 31.5°C, 48 h	11.8	32.8	–	[58]
B. lactofermentum AJ12937	AEC^{r} and Ala^{-}	Glucose 31.5°C, 58 h	120.5	–	–	[59]

B. lactofermentum AJ11082/pSSM30BS	AEC^r, Ala^-, and CCL^r sucrase gene on plasmid	Sucrose 31.5°C, 35 h	95	–	–	[60]
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	–	[61]
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	–	[62]
B. lactofermentum AJ3990/pHSG::THYB	Transhydrogenase gene on plasmid	Glucose 3.6% 31.5°C, 72 h	14.5	40.3	–	[63]
C. glutamicum AGL-6	Reengineered strain carrying six mutations on genome (*hom59*, *lysC311*, *pyc458*, *gnd361*, *mqo224*, and *leuC456*)	Glucose 40°C, 30 h	100	40	3.3	[64]
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	[65]
C. glutamicum	Classical producer	Glucose 45 h	170	–	3.8	[21]
C. glutamicum Lys5-8	$lysC^{C932T}$, $pyc^{G1A,C1372T}$, hom^{T176C}, $\Delta ilvN_{C-T}$, $\Delta aceE::lysA$, $\Delta alaT::dapA$, $\Delta avtA::ddh$, $\Delta ldhA::dapB$, $\Delta mdh::asd$, $\Delta gapA::gapC$, $\Delta pck::lysC^{C932T}$	Glucose 48 h	130.82	47.06	2.73	[66]
Corynebacterium thermoaminogenes AJ12521	Thermotolerant *Corynebacterium* AEC^r	Glucose 10% 43°C, 72 h	30	30	–	[51]

(continued)

Table 1 (continued)

Strain	Remarks	Culture conditions	Titer (g/L)	Yield (%)	Productivity (g/L/h)	Reference
E. coli W3110*tyrA*/pCABD2	*dapA*, *lysC*, *dapB*, and *ddh* on plasmid	Glucose 4% 37°C, 30 h	12.23	30.6	–	[42]
E. coli AJ13069/pSYBJEl	AEC^r *ybjE* on plasmid	Glucose 4% 37°C, 27 h	7.6	19	–	[46]
E. coli NT1003	Thr^- and Met^- *ppc*, *pntB*, and *aspA* on plasmid	Glucose 72 h	134.9	45.4	1.9	[20]
Bacillus methanolicus NOA2#13A52-8A66	Thermotolerant methylotroph Hse^-, AEC^r, and Lys^r	Methanol 50°C, 60 h	47	–	–	[53]

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

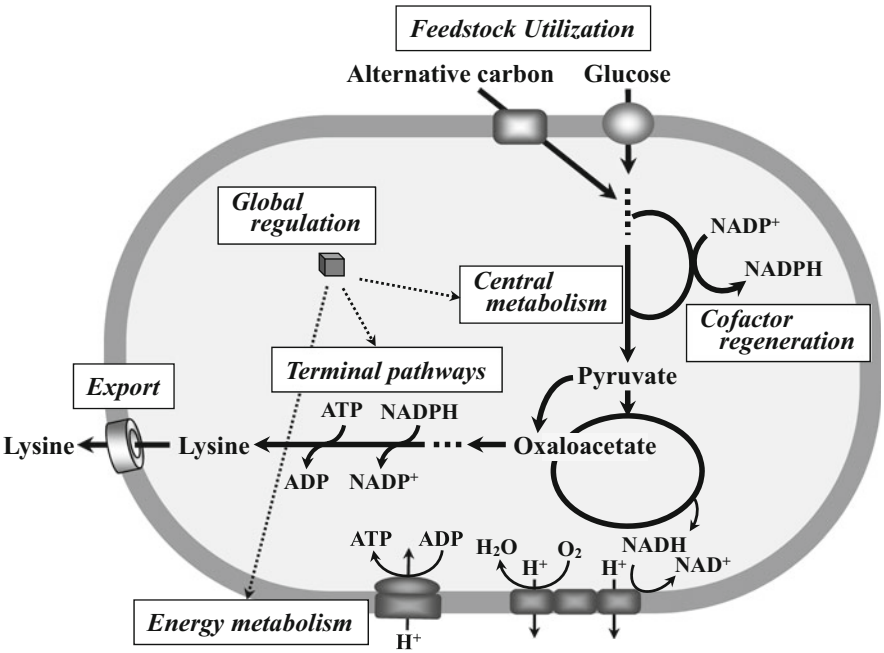

Fig. 2 Targets of metabolic engineering for lysine production

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 [67], by deregulation of phosphoenolpyruvate carboxylase [68], or by deletion of the phosphoenolpyruvate carboxykinase gene [37, 38], resulting in significantly increased production of lysine. Increasing the availability of pyruvate by abolishing pyruvate dehydrogenase activity can also improve lysine production [69]. On the other hand, inactivation or attenuation of the TCA cycle enzyme(s) isocitrate dehydrogenase [39], citrate and methylcitrate synthases [70, 71], or malate:quinone oxidoreductase [72] 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 [73] or through overexpression of the fructose 1,6-bisphosphatase gene [74] or the glucose

6-phosphate dehydrogenase gene [75]. 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 [76]. 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 [77, 78]. 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. 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

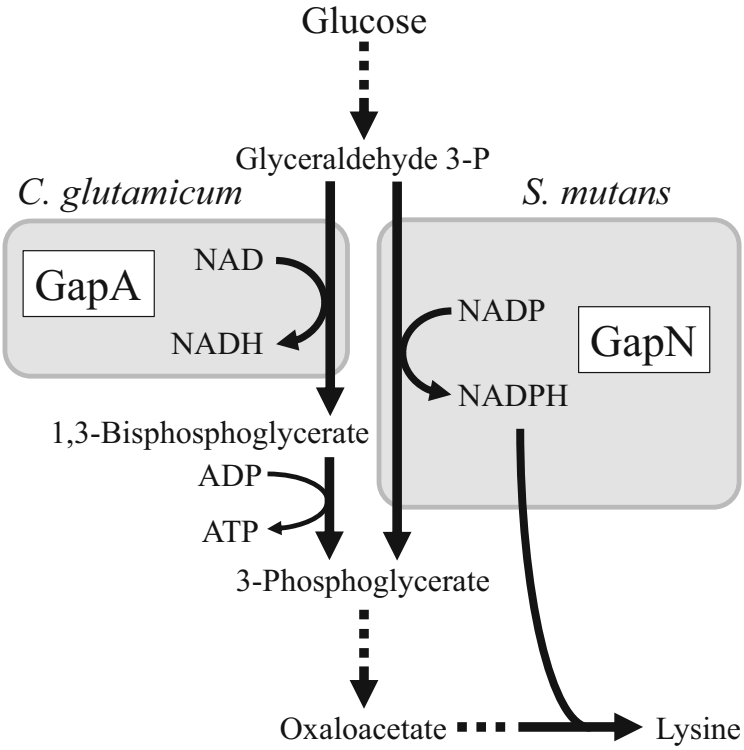

Fig. 3 Design of *S. mutans*-type redox metabolism in *C. glutamicum*. Endogenous NAD-dependent glyceraldehyde 3-phosphate dehydrogenase (*GapA*) of *C. glutamicum* was replaced with nonphosphorylating NADP-dependent glyceraldehyde 3-phosphate dehydrogenase (*GapN*) of *S. mutans*, leading to a *C. glutamicum*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

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 [77]. 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 [79] and the modified endogenous GapA with preference of NADP [80] 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 [81].

3.3 Lysine Export

The export step is critical for achieving efficient amino acid production in *C. glutamicum* [82]. The amino acid exporter first identified in bacteria is LysE, which exports the basic amino acids lysine and arginine in *C. glutamicum* [83]. Overexpression of the *lysE* gene resulted in a fivefold increase in the excretion rate for lysine compared to the rate of the control strain [83]. 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* [84]. 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 [19, 46].

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 [85–89]. These include the *iolT1* and *iolT2* gene products, both known as *myo*-inositol transporters [90]. Expression of the *iolT1*-specific glucose uptake bypass instead of the native PTS resulted in approximately 20% increased lysine production [86]. 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. 4). More

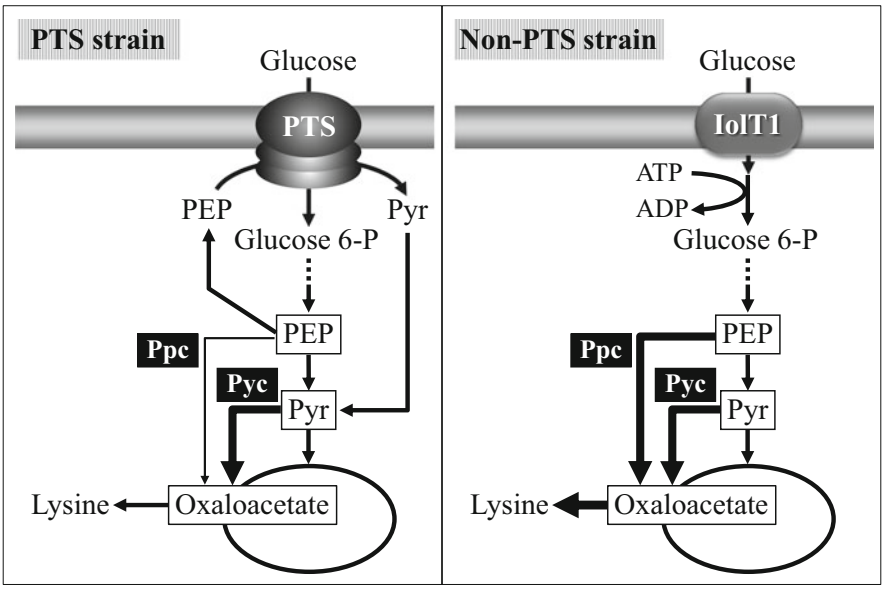

Fig. 4 Possible mechanism for an increase in lysine production, activated by switching the glucose transport systems from the PTS to the *iolT1*-specified non-PTS route. Considering that lysine production is generally limited by the pyruvate carboxylase (*Pyc*) reaction [67], it is likely that the increased 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*)

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 [91]. 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 [92]. 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 [93]. The usefulness of this alteration of the respiratory chain pathway has also been demonstrated for *E. coli* lysine producers [47].

Recently, it has been shown that *C. glutamicum* can grow anaerobically by means of nitrate respiration [94, 95]. 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 [95].

3.6 Global Regulation

Since amino acid biosynthesis in *C. glutamicum* is directly or indirectly subject to both pathway-specific and global regulation [96, 97], 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* [98]. In this strain, the *lysC* gene, encoding the key enzyme aspartokinase, was upregulated severalfolds, 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 [32].

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 toward 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 [99]. *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 [100]. 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 [101]. 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% [102].

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 [103]. Similarly, heterologous expression of the *E. coli* arabinose-utilizing pathway in *C. glutamicum* resulted in a strain that is able to grow on arabinose [104]. 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 [105].

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 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" [15–17, 106–109]. 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). 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.

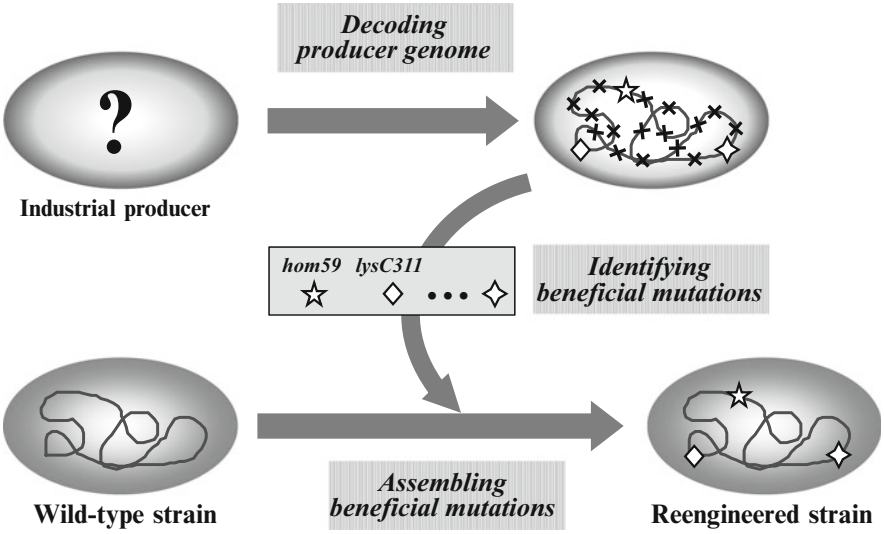

Fig. 5 Reengineering of defined lysine producers by genome breeding. This approach starts with comparative genomic analysis to identify mutations and eventually leads to creation of a defined mutant that carries a minimal set of beneficial mutations (e.g., *hom59*, *lysC311*) and no undesirable mutations (x)

4.1 First Stage: Genome Analysis of a Classical Production Strain

C. glutamicum B-6 (Table 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 [110]. 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. 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 2), revealing that both mutations were basal mutations [108]. 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.

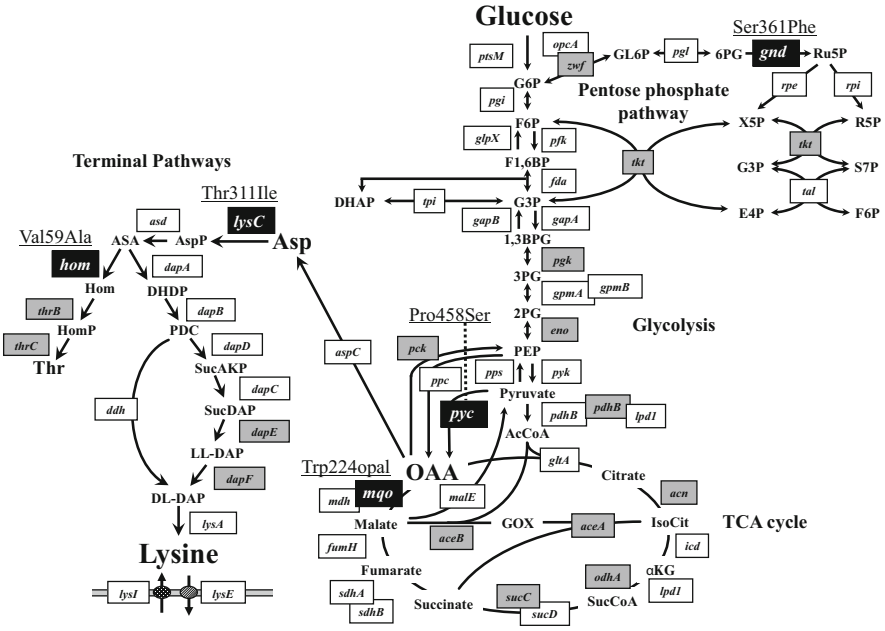

Fig. 6 Mutated genes identified in the lysine-biosynthetic pathways and central carbon metabolism through whole-genome sequencing of *C. glutamicum* B-6. Both *grey* and *black boxes* represent genes in which mutations were identified. The *five specific mutations* indicated above the *black boxes* were defined as positive mutations for lysine production. Abbreviations and symbols for genes are as described in the previous report [111]

Table 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% [15, 108]

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* [112]. Although they are currently classified as belonging to the original species *C. glutamicum* [113], 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 [114]. 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 2) [108]. 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. 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 [108], *gnd361* (S361F) in the pentose phosphate pathway [76], and *mqo224* (W224opal) in the TCA cycle [72], were identified as beneficial mutations for improved production (Fig. 6). Likewise, *leuC456* (G456D) in the leucine-biosynthetic pathway was defined as the beneficial mutation from among mutations in other metabolic pathways [32]. These four beneficial mutations were then assembled one by one under the *hom59* and *lysC311* background, which resulted in stepwise increases in lysine production and a final titer of 100 g/L (Table 2) [15, 64].

4.5 Performance of the Reengineered Strain

The reengineered strain, designated AGL-6 (Table 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 [108]. 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 [15, 115]. 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 [116].

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 [108]. 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 [108]. 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 [76]. 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 downregulation 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 [72]. 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 [32].

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 [11]. 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* [12, 13, 74].

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 [65]. Through a series of these processes, now known as systems metabolic engineering, it has been demonstrated that 12 genetic modifications in a wild-type genome led to the lysine hyperproducer LYS-12 (Table 1, Fig. 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-L jar fermenter cultivation at 30°C. Among the twelve modifications shown in Fig. 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 toward 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

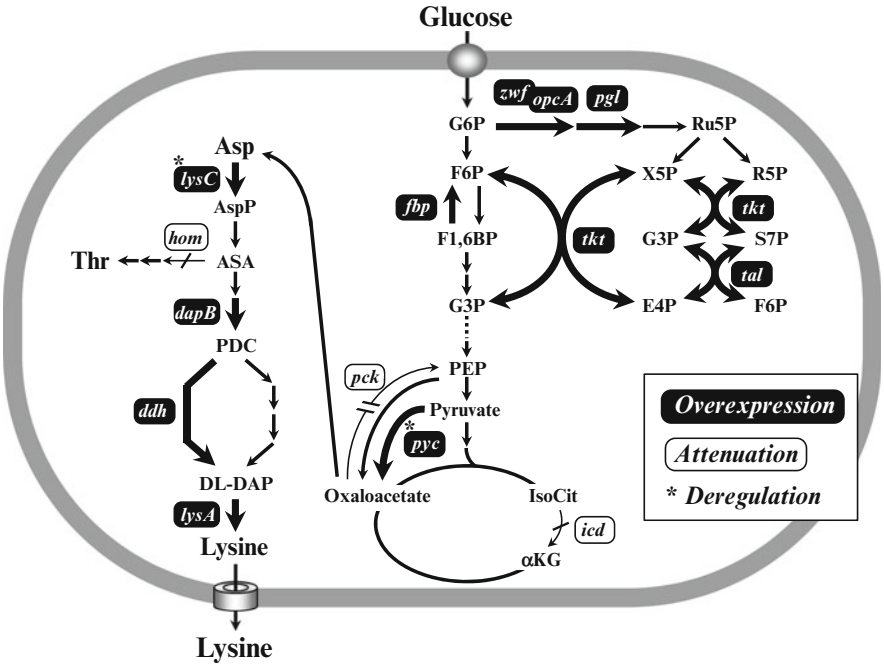

Fig. 7 Systems metabolic engineering for lysine production. On the basis of the metabolic blueprint, 12 genetic modifications were introduced into a wild-type genome, which resulted in the lysine hyperproducer *C. glutamicum* LYS-12. Abbreviations and symbols for genes are as described in the previous report [111]

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 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 [3]. 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 [21]. 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 [117]. 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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Branched-Chain Amino Acids

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Abstract Branched-chain amino acids (BCAAs), viz., L-isoleucine, L-leucine, and L-valine, are essential amino acids that cannot be synthesized in higher organisms and are important nutrition for humans as well as livestock. They are also valued as synthetic intermediates for pharmaceuticals. Therefore, the demand for BCAAs in the feed and pharmaceutical industries is increasing continuously. Traditional industrial fermentative production of BCAAs was performed using microorganisms isolated by random mutagenesis. A collection of these classical strains was also scientifically useful to clarify the details of the BCAA biosynthetic pathways, which are tightly regulated by feedback inhibition and transcriptional attenuation. Based on this understanding of the metabolism of BCAAs, it is now possible for us to pursue strains with higher BCAA productivity using rational design and advanced molecular biology techniques. Additionally, systems biology approaches using augmented omics information help us to optimize carbon flux toward BCAA production. Here, we describe the biosynthetic pathways of BCAAs and their regulation and then overview the microorganisms developed for BCAA production. Other chemicals, including isobutanol, i.e., a second-generation biofuel, can be synthesized by branching the BCAA biosynthetic pathways, which are also outlined.

Keywords Branched-chain amino acids (BCAAs), *Corynebacterium glutamicum*, *Escherichia coli*, Feedback inhibition, Isobutanol, L-Isoleucine, L-Leucine, L-Valine, Metabolic engineering, Transcriptional attenuation

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Contents

1 Introduction

Branched-chain amino acids (BCAAs), namely, L-isoleucine, L-leucine, and L-valine, are essential amino acids [1] that are not synthesized in mammals, but have critical roles in physiological functions and metabolism [2]. BCAAs are used in dietary products, pharmaceuticals, and cosmetics and serve as a precursor of antibiotics and herbicides. Moreover, they are expected to play a leading role in future feed additives [3]. As other amino acids, BCAAs have been manufactured by fermentation using mutated or metabolically engineered microorganisms that originated from *Corynebacterium glutamicum* or *Escherichia coli* [4, 5]. The amount of the annual production of L-isoleucine, L-leucine, and L-valine in 2001 was approximately 400, 500, and 500 tons, respectively [6, 7], and has been increasing continuously. For example, the nonfeed market for L-valine reached around 1,000–1,500 tons per annum with an estimated 5–8 % annual increase [8].

Historically, BCAAs were produced chemically, and their enantiomers were separated enzymatically after chemical derivatization or via chromatographic separation followed by crystallization. However, as the demand for BCAAs increased, fermentative methods gathered attention for economic reasons as well as from an environmental perspective. In the early stages, most BCAA production strains were isolated by random mutagenesis. However, the random mutagenesis approach distributes genetic alterations throughout the chromosome, which are difficult to identify and may cause unexpected effects. Recently, amino acid-producing strains have been developed by rational genetic manipulation to avoid this problem. Highly productive strains can be created in a genetically defined manner, e.g., by specifically overexpressing biosynthetic genes responsible for a target amino acid. Systems metabolic engineering, which analyzes metabolism in a genome scale [9], also helps us to optimize carbon flux toward the biosynthesis of a target amino acid [10].

In this chapter, we will describe the biosynthetic pathways of BCAAs and their regulation in microorganisms, in particular, *C. glutamicum*, which is a nonpathogenic Gram-positive bacterium of the family Actinomycetes and is widely used for the industrial production of amino acids and nucleotides [11]. The development of

microbial strains for production of BCAAs will then be outlined. Finally, the extrapolated application of the BCAA biosynthetic pathways to other chemicals, including isobutanol, i.e., a second-generation biofuel, will be shown.

2 Regulation of BCAA Biosynthesis

The biosynthetic pathways of BCAAs of *C. glutamicum* are summarized in Fig. 1. *C. glutamicum* has one acetohydroxy acid synthase (AHAS) encoded by *ilvBN* [12], which catalyzes the initial step of the BCAA biosynthesis. AHAS produces

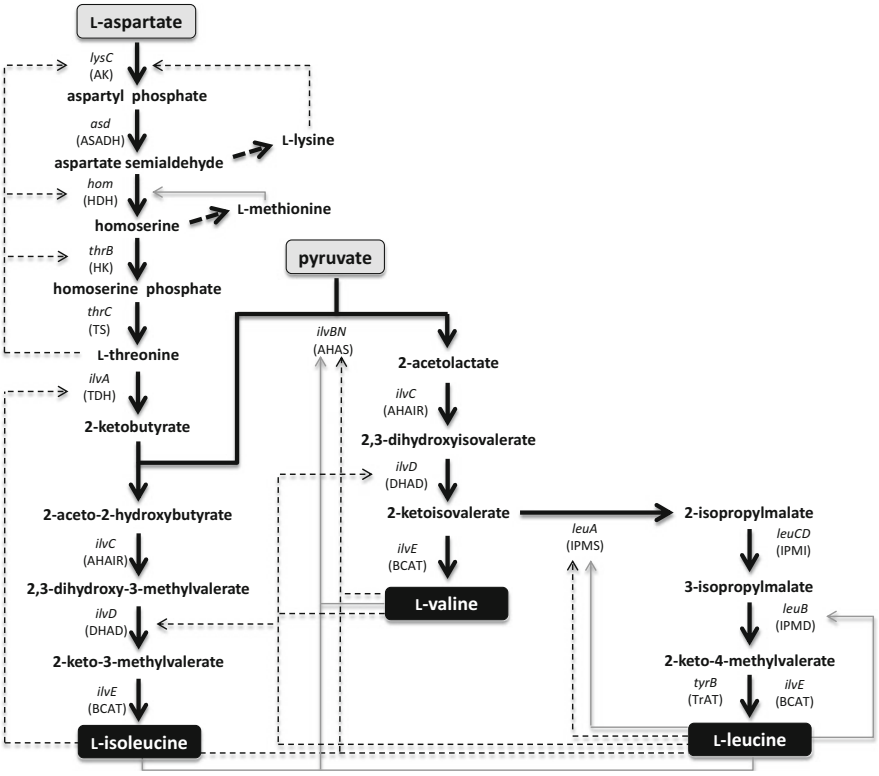

Fig. 1 The BCAA biosynthetic pathways and their regulation in *C. glutamicum*. The genes and enzymes are shown in *italic font* and in *parentheses*, respectively. *Dotted lines* and *gray lines* indicate feedback inhibition and transcriptional attenuation, respectively. Abbreviations: *AHAIR* acetohydroxy acid isomeroreductase, *AHAS* acetohydroxy acid synthase, *AK* aspartate kinase, *ASADH* aspartate semialdehyde dehydrogenase, *BCAT* branched-chain amino acid aminotransferase, *DHAD* dihydroxy acid dehydratase, *HDH* homoserine dehydrogenase, *HK* homoserine kinase, *IPMD* isopropylmalate dehydrogenase, *IPMI* isopropylmalate isomerase, *IPMS* isopropylmalate synthase, *TDH* threonine dehydratase, *TrAT* tyrosine-repressible transaminase, *TS* threonine synthase

2-acetolactate and 2-aceto-2-hydroxybutyrate for the L-leucine/L-valine and L-isoleucine biosynthesis, respectively. The starting materials for this reaction are pyruvate and 2-ketobutyrate. Pyruvate is provided from the glycolytic pathway, while 2-ketobutyrate is synthesized from L-threonine by threonine dehydratase (TDH) encoded by *ilvA*. TDH is feedback inhibited by L-isoleucine [13], although the activity can be restored by L-valine [14]. AHAS consists of large and small subunits, which are encoded by *ilvB* and *ilvN*, respectively. The small subunit of AHAS is responsible for the multivalent regulation by all three BCAAs [12]. Unlike *C. glutamicum*, some bacteria have several isoforms of AHAS. For instance, *E. coli* has three isozymes, AHAS I, II, and III [3], which are encoded by *ilvBN*, *ilvGM*, and *ilvIH*, respectively. The expression of these genes is regulated differently, and *ilvGM* is attenuated by all BCAAs, while *ilvBN* is affected only by L-leucine and L-valine [15]. The activity of AHAS I and III is inhibited strongly by L-valine and weakly by L-isoleucine, while L-leucine has no effect [16]. *E. coli* is very sensitive to L-valine, and its growth is inhibited at an extremely low concentration [17].

The *ilvC* gene encodes acetohydroxy acid isomeroreductase (AHAIR) and is transcripted as an operon with the *ilvBN* genes [18] in *C. glutamicum*. AHAIR converts 2-acetolactate to 2,3-dihydroxyisovalerate in the L-valine and L-leucine biosynthesis and 2-aceto-2-hydroxybutyrate to 2,3-dihydroxy-3-methylvalerate in the L-isoleucine biosynthesis by using NADPH as a cofactor. The expression of *ilvBNC* operon is controlled by transcriptional attenuation, which is mediated by all BCAAs [19].

Dihydroxy-acid dehydratase (DHAD) encoded by *ilvD* [20] is the enzyme for the next biosynthetic step in which 2-ketoisovalerate and 2-keto-3-methylvalerate are formed from 2,3-dihydroxyisovalerate and 2,3-dihydroxy-3-methylvalerate, respectively. This enzyme is inhibited by either L-valine or L-leucine [21]. Transcriptional regulation of the *ilvD* gene is unknown.

Branched-chain amino acid transaminase (BCAT) or transaminase B encoded by *ilvE* [20] is the last player in the L-isoleucine and L-valine biosynthesis. This enzyme transfers the amine moiety of L-glutamate to 2-ketoisovalerate and 2-keto-3-methylvalerate to afford L-valine and L-isoleucine, respectively [22].

The specific pathway of the L-leucine biosynthesis starts from the reaction by isopropylmalate synthase (IPMS) encoded by *leuA*, which generates 2-isopropylmalate from 2-ketoisovalerate and acetyl-CoA. This enzyme in *C. glutamicum* is subjected to strong feedback inhibition, and its expression is also regulated by L-leucine [23]. Then, 2-isopropylmalate is isomerized to 3-isopropylmalate by isopropylmalate isomerase (IPMI). IPMI consists of large and small subunits, which are encoded by *leuC* and *leuD*, respectively. Next, 3-isopropylmalate is converted to 2-keto-4-methylvalerate by isopropylmalate dehydrogenase (IPMD), which is encoded by *leuB*. In *C. glutamicum*, *leuB* is strongly repressed by L-leucine [24], while the *leuABCD* genes form an operon in *E. coli*, and their expression is controlled by the transcriptional attenuation mediated by L-leucine [25]. As the other two BCAAs, L-leucine is formed from 2-keto-4-methylvalerate by the catalysis of BCAT, which is activated by the substrate itself

[26]. Additionally, the same reaction is catalyzed by tyrosine-repressible transaminase (TrTA) encoded by *tyrB* [27].

3 Production of BCAAs by Metabolically Engineered Microorganisms

In this section, we describe the BCAA-producing strains that have been developed to date. Table 1 shows the recent representative strains for BCAA production.

3.1 L-Valine

Microbial production of L-valine was first reported by Udaka and Kinoshita [50] and Sugisaki [51] independently. Udaka and Kinoshita screened a large number of microorganisms, and the selected bacteria, namely, *Paracolobacterum coliforme* and *Brevibacterium ammoniagenes*, produced L-valine in 23 % molar yield from glucose. Sugisaki isolated *Aerobacter cloacae* and *A. aerogenes*, which produced L-valine in 20 % molar yield from glucose. These initial findings provoked a hunt for better L-valine producers, and auxotrophic mutants or amino acid analog-resistant mutants were included within the research scope. One example of the auxotrophic mutants is the isoleucine auxotrophic mutant of *Micrococcus glutamicus* [52], which produced more than 10 g/L L-valine when the culture was supplemented with a small amount of DL-isoleucine and DL-valine. An amino acid analog-resistant mutant accumulating L-valine was then found while validating a method to isolate amino acid-producing microorganisms using *E. coli* ATCC 4157 as a model microbe [53]. This L-valine-producing mutant was originated from a norvaline-resistant strain, which was further mutated to acquire L-leucine auxotrophy. The resultant strain accumulated more than 2 g/L L-valine. An amino acid analog-resistant mutant was also developed using *Serratia marcescens* [54]. Among several BCAA analogs tested, α-aminobutyric acid conferred mutants that were able to accumulate more than 8 g/L L-valine. An enzymatic analysis revealed that in these mutants, expression of AHAS was derepressed and/or AHAS was only weakly affected by feedback inhibition. Three L-glutamate-producing bacteria, *B. lactofermentum*, *C. acetoacidphilum*, and *Arthrobacter citreus*, were also led to L-valine production mutants using a histidine analog, 2-thiazolealanine [55]. The most efficient strain, one from *B. lactofermentum*, achieved 31 g/L production in 72 h. In this strain also, AHAS was desensitized from the feedback inhibition by L-valine as well as L-isoleucine and L-leucine. Moreover, the expression of this enzyme was partially derepressed [56]. An interesting example is the α-aminobutyric acid-resistant mutant of the biotin-auxotrophic *B. flavum* MJ-233 [57]. In the reaction using this mutant, carbon resources were converged to L-valine

Table 1 The representative strains for production of BCAAs

BCAA	Parent strain	Genotype	Plasmid	Reaction type	Final conc. (mM)	Yield (mol% of glucose)	Comment	Reference
L-Valine	*C. glutamicum* ATCC13032	Δ*ilvA* Δ*panBC*	pJC1ilvBNCD	Flask	92			[20]
		Δ*ilvA* Δ*panB ilvN*M13	pECKAilvBNC	Flask	130			[28]
		Δ*panB* *ilvN*M13 P-*ilvA*M1CG P-*ilvD*M7 P-*ilvE*M6		Flask	136			[29]
		MP*ilvA* Δ*avtA*	pDXW-8-*ilvEBN*^{r}*C*	Fed-batch	266	27	15 % saturation of DO	[30]
		*atpG*DC*	pVKilvN53C	Flask	96			[31]
		Δ*aceE*	pJC41ilvBNCE	Fed-batch	210	60	KOAc supplementation	[32]
		Δ*aceE* Δ*pqo*	pJC41ilvBNCE	Fed-batch	210	23	KOAc supplementation	[33]
		Δ*aceE* Δ*pqo*	pJC41ilvBNCE	Fed-batch	301	20	EtOH supplementation	[33]
		Δ*aceE* Δ*pqo* Δ*sugR*	pJC41ilvBNCE	Fed-batch	160	17		[33]
		Δ*aceE* Δ*pqo* Δ*pgi*	pJC41ilvBNCE	Fed-batch	412	75	KOAc supplementation	[34]
	C. glutamicum R	Δ*ldhA*	pCRB-BN^{GE}C^{TM} pCRB-DLD	Fed-batch	1,470	63	Oxygen deprivation condition	[35]
		Δ*ldhA* Δ*pta* Δ*ackA* Δ*ctfA* *ilvN*^{GE}*C*^{TM} *gapA*, *pyk*, *pfkA*, *pgi*, *tpi*	pCRB-BN^{GE}C^{TM} pCRB-DLD	Fed-batch	1,280	88	Oxygen deprivation condition	[36]

	C. glutamicum ATCC13869	Δ*aceE* Δ*ilvA* Δ*alaT*	pJYW-4-*ilvBNC*$_1$-*lrp*$_1$-*brnFE*	Fed-batch	437	47		[37]
	B. flavum JV16	*avtA*::*Cm*		Fed-batch	331	39		[30]
	B. flavum ATCC14067		pDXW-8-*ilvEBN*^{r}*C*	Fed-batch	325	37	High temperature (37°C)	[38]
	E. coli W3110	*ilvH*(G41A C50T) P_{tac}-*ilvBN*, P_{tac}-*ilvGMED* Δ*ilvA* Δ*panB* Δ*leuA* Δ*aceF* Δ*pfkA* Δ*mdh*	pKBRilvBNCED pTrc184ygaZHlrp	Fed-batch	64	58		[39]
L-Isoleucine	*C. glutamicum* ATCC 21799		pGC77 (*hom*^{dr}, *thrB*, *ilvA*)	Flask	114			[40]
			pAPE18 (*hom*^{dr}, *thrB*, *tdcB*)	Flask	30			[41]
	C. glutamicum MH20-22B	*hom*(Fbr) × 3 copies *thrB* × 4 copies	pECM3-*ilvA* (H278R-L351S)	Flask	96			[13]
	C. glutamicum MH20-22B		pECM3-*ilvA* (V323A) pEK-*hom*(Fbr)-*thrB*	Flask	63			[42]
	C. glutamicum YILW	Δ*alaT*	pXMJ19-*thrABC*	Fed-batch	100			[43]
	C. glutamicum JHI3-156			Batch	203	27	Optimized DO and pH control	[44]
			pDXW-8-*ilvBNC1-ilvA1*	Fed-batch	234	16		[45]
			pDXW-8-*lrp-brnFE*	Fed-batch	205	17		[46]

(continued)

Table 1 (continued)

BCAA	Parent strain	Genotype	Plasmid	Reaction type	Final conc. (mM)	Yield (mol% of glucose)	Comment	Reference
	C. glutamicum WJ001		pDXW-8-*fusA-frr-ilvBNA-ppnk*	Fed-batch	217	19		[47]
	E. coli K-12 TDH6		pVICLC80A (*thrA*BC*, *lysC**) pMWD5 (*ilvGMEDA**)	Flask	94			[48]
L-Leucine	*C. glutamicum* ATCC13032	P_{tuf}-*leuA*_B018 Δ*leuA*::P_{tuf}-*leuA*_B018 Δ*ltbR*::P_{tuf}-*leuA*_B018 $P_{dap\text{-}L1}$-*gltA*, *ilvN*_fbr, Δ*iolR*		Fed-batch	152	26		[49]

production when the medium lacked biotin and the bacteria did not grow [58]. This "living cell reaction" process using this strain allowed for accumulation of 300 mM L-valine within one day in 80 % molar yield from glucose with 96 % purity out of the total amino acids.

Genetically defined strains for L-valine production have been developed mostly by using *C. glutamicum* along with a few examples using other bacteria, including *E. coli*. The following mainly describes the strains derived from these two bacteria.

The general basic strategy in fermentative production of chemicals is to direct common intermediates for various end products solely to a target compound. In the case of L-valine, this can be achieved by deleting or repressing the transcription of *ilvA* and *panB* [20, 28–30, 59], which are the first genes for the L-isoleucine and D-pantothenate biosynthesis, respectively (Figs. 1 and 2). Limitation of D-pantothenate biosynthesis leads to reduction of CoA supply, which is an additional benefit for L-valine production because consumption of pyruvate by pyruvate dehydrogenase complex (PDHC) is suppressed due to the decreased concentration of the reaction partner [20].

Radmacher et al. [20] prepared *C. glutamicum* Δ*ilvA* Δ*panBC* whose biosynthesis of L-valine was strengthened by introduction of the plasmid containing *ilvBNCD* or *ilvBNCE*. The best strain *C. glutamicum* Δ*ilvA* Δ*panBC* (pJC1ilvBNCD) accumulated 92 mM L-valine along with a small amount of L-alanine (1.3 mM) in 48 h. Though AHAS was not desensitized from the feedback

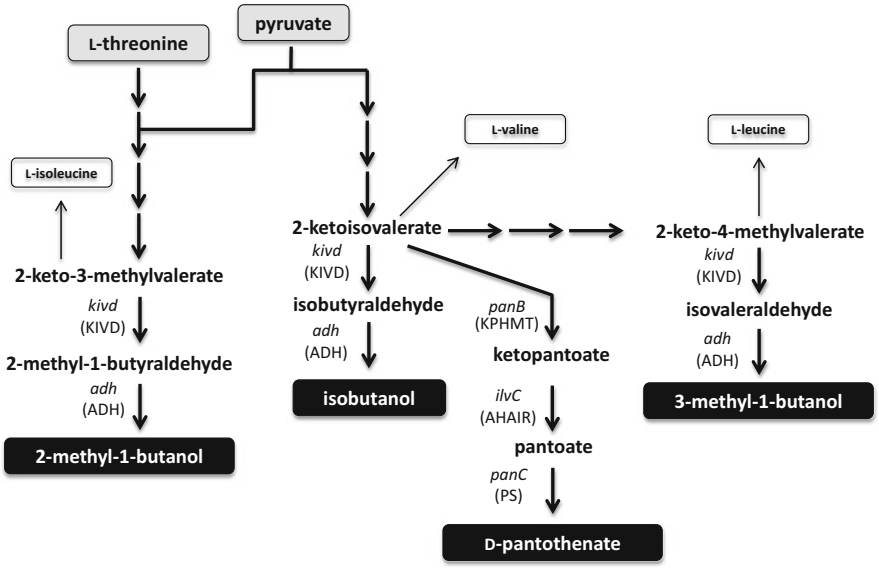

Fig. 2 The biosynthetic routes branched from the BCAA biosynthetic pathways for production of isobutanol, 3-methyl-1-butanol, 2-methyl-1-butanol, and D-pantothenate. Abbreviations: *ADH* alcohol dehydrogenase, *AHAIR* acetohydroxy acid isomeroreductase, *KIVD* 2-ketoisovalerate decarboxylase, *KPHMT* ketopantoate hydroxymethyltransferase, *PS* pantothenate synthetase

inhibition by L-valine in this strain, it was able to produce L-valine efficiently. This is because the feedback inhibition of AHAS of *C. glutamicum* by L-valine is not very strong, and the maximum inhibition of the enzymatic activity does not exceed 50 % [12]. Nonetheless, desensitization of AHAS from the feedback inhibition was investigated [28]. The native gene of the regulatory subunit of AHAS (*ilvN*) was replaced with the feedback-resistant mutant *ilvN*M13, which was designed based on the precedent structural information on the feedback-resistant homologs of *E. coli* and *Streptomyces cinnamonensis*. This plasmid-free strain *C. glutamicum* Δ*ilvA* Δ*panB ilvN*M13 produced 90 mM L-valine in the 48-h cultivation, while less than 40 mM L-valine formed in the culture of the parent strain *C. glutamicum* Δ*ilvA* Δ*panB*. L-Valine production by these strains was strengthened by a plasmid-carrying *ilvBNC*, and the resultant strain *C. glutamicum* Δ*ilvA* Δ*panB ilvN*M13 (pECKAilvBNC) produced 130 mM L-valine. It should be mentioned that the effect of the mutation on *ilvN* was less significant in the strains with the plasmid-borne *ilvBNC*, and the parent *C. glutamicum* Δ*ilvA* Δ*panB* (pECKAilvBNC) was able to produce up to 120 mM L-valine.

Formation of L-alanine was a prevailing issue in the L-valine-producing strains [59]. Pyruvate is a common precursor for the biosynthesis of L-valine and L-alanine. Therefore, suppression of L-alanine formation is beneficial for L-valine production. Two genes, *alaT* or *avtA*, are responsible for the conversion of pyruvate to L-alanine, which encode aminotransferases using L-glutamate or L-valine as an amine source, respectively [59]. Deletion of either gene was performed on *C. glutamicum* Δ*ilvA* Δ*panBC* (pJC1ilvBNCD), and there was no detrimental effect on L-valine production in both cases. While the *avtA*-deficient mutant showed only slight decrease of L-alanine accumulation, the *alaT*-deficient mutant improved greatly, and L-alanine formation decreased to 0.16 mM from 1.2 mM in the parent strain. It should be mentioned that the effect of deletion of these genes may be dependent on strains, and in other cases, effectiveness of *avtA* deletion has been demonstrated as described below.

In place of the complete deletion of the side-path genes or strong overexpression of the rate-limiting enzymes for production of target compounds, their expression can be modified by tuning promoter activity. Holátko et al. [29] prepared *C. glutamicum ilvN*M13 Δ*panB* P-*ilvA*M1CG P-*ilvD*M7 P-*ilvE*M6, in which expression of *ilvA* was downregulated by the mutated promoter, while that of *ilvD* and *ilvE* was upregulated. This strain produced 136 mM L-valine in 48 h of flask fermentation. Modulation of promoter activities allows for overexpression of genes without using plasmids, which ensures genetic stability as well as no need for antibiotic markers. Additionally, the bradytrophic property is advantageous in that the strains do not require nutrition supplementation.

Recently, a strain with mixed strategies was reported [30], where expression of *ilvA* is partially limited by a mutated weak promoter, and production of L-alanine was suppressed by deletion of *avtA*. The resultant strain overexpressing the feedback-free L-valine biosynthetic genes, *C. glutamicum* MP*ilvA* Δ*avtA* (pDXW-8-*ilvEBN*^{r}*C*), produced 266 mM L-valine in 27 % molar yield from glucose. It is noteworthy that the side products, such as L-lactate and L-glutamate, were

controlled by maintaining the dissolved oxygen (DO) at 15 % saturation. The same strategy was applied to *B. flavum* JV16, which is an α-aminobutyric acid-resistant and Leu–Ile–Met-auxotrophic strain generated by random mutagenesis from *B. flavum* DSM20411. The resultant strain *B. flavum* JV16 *avtA*::*Cm* (pDXW-8-*ilvEBN*^{r}*C*) produced 331 mM L-valine in 39 % molar yield from glucose [30]. It should be mentioned that there is another example of an L-valine-producing strain using this subspecies of *C. glutamicum* [38]. *B. flavum* ATCC14067, which was transformed with the plasmid pDXW-8-*ilvEBN*^{r}*C*, was able to produce 325 mM L-valine in 37 % molar yield from glucose at elevated temperatures as high as 37°C after the 48-h fed-batch fermentation.

An alternative strategy for producing L-valine to deleting *ilvA* and *panB* was reported by Blombach et al. [32]. Deletion of *aceE*, which encodes the E1p subunit of PDHC, resulted in inability to grow solely on glucose, while acetate supplementation compensated for it [60]. Blombach et al. [32] investigated accumulation of organic acids and amino acids in the culture medium of this strain and found that it started to produce pyruvate (30–35 mM), L-alanine (25–30 mM), and L-valine (30–35 mM) after depletion of acetate. Pyruvate accumulated as a direct consequence of inactivation of PDHC, while L-alanine and L-valine were the drain-off compounds of pyruvate. When *ilvBNCE* was overexpressed using the plasmid, the resultant strain *C. glutamicum* Δ*aceE* (pJC4ilvBNCE) produced 210 mM L-valine in the overall 50 % molar yield from glucose along with 5 mM pyruvate in the fed-batch process. This strain is advantageous over the aforementioned Δ*ilvA* Δ*panB* strains because it does not require supplementation of L-isoleucine or D-pantothenate.

Blombach et al. [34] performed further improvement of this *aceE*-deficient strain. They deleted the *pqo* gene encoding pyruvate:quinone oxidoreductase (PQO), which converts pyruvate to acetate and carbon dioxide. This resulted in an increase of 30 % molar yield. It is likely that PQO in the combination with acetate kinase and phosphotransacetylase bypasses the PDHC reaction to provide acetyl-CoA when the cell density is high. Therefore, inactivation of PQO led to the increase of L-valine production by cutting off the supply of the carbon resources for growth purposes in the late phase. They continued to observe pyruvate in the culture of *C. glutamicum* Δ*aceE* Δ*pqo* (pJC4ilvBNCE), which indicated that L-valine production is limited by the downstream reactions from pyruvate to L-valine. They then tested to improve supply of NADPH. In the total reactions from glucose to L-valine, the downstream part (pyruvate to L-valine) requires two equivalents of NADPH at the AHAIR reaction as well as for regeneration of L-glutamate consumed by BCAT, whereas the glycolytic pathway provides only NADH, not NADPH. To compensate for the shortage of NADPH supply in L-valine production, they deleted *pgi* so that the carbon flux from glucose is directed to the pentose phosphate pathway, which produces 2 mol of NADPH from 1 mol of glucose. This strain, *C. glutamicum* Δ*aceE* Δ*pqo* Δ*pgi* (pJC4ilvBNCE), achieved more than 400 mM L-valine excretion in the 75 % molar yield from glucose, and pyruvate was not observed anymore. From the viewpoint of carbon usage, the deletion of *pyc* encoding pyruvate carboxylase was also beneficial, and *C. glutamicum* Δ*aceE*

Δ*pqo* Δ*pgi* Δ*pyc* (pJC4ilvBNCE) reached 86 % molar yield, although the final concentration of L-valine was about 240 mM.

Addition of acetate inhibits production of L-valine during growth of the Δ*aceE* strains because acetate represses the genes of the phosphoenolpyruvate:sugar phosphotransferase system (PTS), *ptsG*, *ptsI*, and *ptsH* via the DeoR-type regulator, SugR, and prevents uptake of glucose [61–63]. Therefore, Blombach et al. [33] deleted the *sugR* gene to remove repression of the PTS genes. Indeed, *C. glutamicum* Δ*aceE* Δ*pqo* Δ*sugR* (pJC4ilvBNCE) consumed glucose five times faster than the parent strain and produced L-valine even in the growth phase in the presence of acetate, although the overall production was 40 % lower. Alternatively, they tested ethanol instead of acetate as the secondary carbon source to avoid the repression of the PTS genes. Under this condition, *C. glutamicum* Δ*aceE* Δ*pqo* (pJC4ilvBNCE) was able to produce L-valine during its growth phase. These strains are effective producers of L-valine, but they accumulated 14–26 mM L-alanine and pyruvate. This accumulation of the by-products may be overcome by supply of NADPH in the strategy mentioned above.

Recently, Chen et al. [37] prepared a strain, which lacked both *ilvA* and *aceE* and *alaT* from *C. glutamicum* ATCC13869 to direct as much pyruvate as possible for production of L-valine. They also overexpressed the *brnF* and *brnE* genes encoding the BCAA exporter and the *lrp* gene encoding the global regulator Lrp that activates the expression of *brnFE* in addition to the L-valine biosynthetic genes (*ilvBNC*). The resultant strain *C. glutamicum* ATCC13869 Δ*aceE* Δ*alaT* Δ*ilvA* (pJYW-4-*ilvBNC*$_1$-*lrp*$_1$-*brnFE*) produced 435 mM L-valine after a 96-h fermentation under the fed-batch condition.

Other strains of interest are the H^+-ATPase-defective strains [31], which are known to increase the intracellular concentration of pyruvate [64, 65]. The native *atpGDC* genes were replaced with the inactivated *atpG*DC* genes containing a single point mutation, and the resultant strain was transformed with the plasmid pVK7ilvN53C, which allows for overexpression of feedback-resistant AHAS as well as AHAIR. *C. glutamicum atpG*DC* (pVK7ilvN53C) produced 96 mM L-valine in 72 h under the flask-shaking condition.

A completely different strategy is to exploit oxygen deprivation conditions, where most of the glucose is expected to be utilized for product formation and not for growth in *C. glutamicum*. Hasegawa et al. [35] used the strain without the *ldhA* gene, which encodes lactate dehydrogenase and is responsible for the production of lactate, i.e., the main fermentation product under oxygen deprivation conditions. Mere overexpression of the L-valine biosynthetic *ilvBNCDE* genes did not result in the efficient L-valine production because of poor glucose uptake caused by the redox imbalance. They solved this issue by converting the cofactor dependence from NADPH to NADH through mutagenesis of AHAIR (*ilvC*^{TM}) and introduction of NADH-dependent exogenous leucine dehydrogenase in place of NADPH-dependent endogenous BCAT using the plasmid, pCRB-DLD. In addition, the feedback-resistant mutant of AHAS (*ilvBN*^{GE}) was overexpressed. The

resultant strain *C. glutamicum* R Δ*ldhA* (pCRB-BN^{GE}C^{TM}, pCRB-DLD) produced 1,470 mM[1] L-valine in 63 % molar yield from glucose in 24 h, and the concentration of L-valine reached 1,940 mM[1] in 48 h. This strain produced succinate as a major by-product and left room for improvement [36]. To minimize the carbon flux to succinate, the phosphoenolpyruvate carboxylase gene *ppc* was deleted. While succinate production was suppressed, this resulted in the elevated NADH/NAD$^+$ ratio. However, this redox imbalance was overcome by deletion of three genes, *ctfA*, *pta*, and *ackA*, associated with acetate synthesis, which produces excess NADH. Additionally, five glycolytic genes, *gapA*, *pyk*, *pfkA*, *pgi*, and *tpi*, were overexpressed. Moreover, L-alanine production was suppressed by deleting *avtA*. The resultant strain *C. glutamicum* R *ilvN*^{GE}*C*^{TM}, *gapA*, *pyk*, *pfkA*, *pgi*, and *tpi* Δ*ldhA* Δ*ppc* Δ*pta* Δ*ackA* Δ*ctfA* Δ*avtA* (pCRB-BN^{GE}C^{TM}, pCRB-DLD) produced 1,280 mM[1] L-valine in 88 % molar yield from glucose in the 24-h fed-batch fermentation.

Compared with *C. glutamicum*, development of the L-valine-producing strains of *E. coli* lags behind, which is probably because of the more complicated regulatory mechanisms for L-valine biosynthesis. However, using *E. coli* as a base strain is advantageous because of its rapid growth rate and rich genetic information. Early examples include the strains that acquire resistance against the feedback inhibition of AHAS III [66] or overexpress *ygaZH* encoding the L-valine exporter [67]. The strain with the mutation in isoleucine-tRNA synthetase [68] and the lipoic acid auxotroph with the inactivated H$^+$-ATPase [69] were also reported to accumulate L-valine. However, these strains were prepared by classical random mutagenesis and their genotypes cannot be defined. It was only recently that the rationally designed strain of *E. coli* was reported to produce L-valine [39]. In this example, Park et al. first removed the product regulation by disarming the feedback inhibition of AHAS III and removing the transcriptional attenuation of the *ilvBN* and *ilvGMEDA* operons by replacing their attenuator leader regions with *tac* promoters. Then the *ilvA*, *panB*, and *leuA* genes were knocked out to converge the carbon resources to L-valine production. Next, AHAS I, which has higher affinity to pyruvate than other two isozymes, was overexpressed as well as *ilvCED* by the plasmid pKBRilvBNCED. The strain was further improved by the plasmid pTrc184ygaZHlrp harboring *lrp* and *ygaZH* that encodes the positive regulator for the *ilvIH* operon and the L-valine exporter, respectively. In silico gene knockout simulation was then performed, and they identified three candidates to be deleted: *aceF*, *pfkA*, and *mdh*. When these genes were deleted, the resultant strain *E. coli ilvH* (G41A, C50T), P*tac-ilvBN*, P*tac-ilvGMED*, and Δ*ilvA* Δ*panB* Δ*leuA* Δ*aceF* Δ*pfkA* Δ*mdh* (pKBRilvBNCED, pTrc184ygaZHlrp) improved the yield and produced 64 mM L-valine in 58 % molar yield from glucose.

[1]The concentration values are corrected by the dilution factors caused by addition of ammonia solution to maintain the pH of the reaction solutions.

3.2 L-Isoleucine

An L-isoleucine producer was initially reported by Hayashibe and Uemura [70], which is an α-aminobutyric acid-resistant *B. subtilis* No. 14 that was isolated during the investigation of threonine metabolism and produced 4.3 g/L L-isoleucine. Screening of the α-aminobutyric acid-resistant microorganisms led to other L-isoleucine-producing strains, such as *A. aerogenes* IAM 1019 (2.4 g/L), *Pseudomonas aureofaciens* IAM 1001 (3.0 g/L), *S. marcescens* (2.7 g/L), and *Erwinia carotovora* E30 (1.3 g/L) [70]. Alternatively, D-threonine was used as a natural amino acid analog for the screening of the bacteria belonging to genera *Serratia* and *Pseudomonas* [71]. The most efficient strain among them, *S. marcescens* No. 1, produced more than 8 g/L L-isoleucine in a 40-h incubation. More microorganisms for L-isoleucine production were isolated using other BCAA analogs [72], including thiaisoleucine for *E. coli* [73, 74], *Salmonella typhimurium* [72, 75], and *Saccharomyces cerevisiae* [76], cyclopentaneglycine for *S. typhimurium* [75], glycyl isoleucine for *E. coli* [74], isoleucine hydroxamate for *S. marcescens* [77], and ketomycin for *Bacillus subtilis* [78]. As is the case for L-valine production, the "living cell reaction" process was effective for L-isoleucine production [58, 79, 80], which was performed using an α-aminobutyric acid-resistant *B. flavum* MJ-233 [57] to produce 200 mM L-isoleucine per day.

For rational metabolic engineering to enhance L-isoleucine production, it is important to address the fact that the biosynthetic pathway of L-isoleucine shares the genes and enzymes with those of L-valine and L-leucine (Fig. 1) and to converge the common intermediates toward the L-isoleucine synthesis. The specific issue for L-isoleucine production is the supply of L-threonine, which is one of the precursors for L-isoleucine biosynthesis. Additionally, removal of the tight feedback regulation for TDH (Fig. 1) is key to the efficient production of L-isoleucine.

As mentioned above, L-isoleucine production requires a supply of L-threonine. Therefore, the biosynthetic pathway of L-threonine and its regulation will be outlined briefly (Fig. 1). The biosynthesis of L-threonine starts from L-aspartate, and the pathway consists of five enzymatic reactions [81], which are catalyzed by aspartate kinase, aspartate semialdehyde dehydrogenase, homoserine dehydrogenase, homoserine kinase, and threonine synthase. *E. coli* has three aspartate kinase isozymes, I, II, and III, encoded by *thrA*, *metL*, and *lysC*, respectively, while *C. glutamicum* has only one aspartate kinase encoded by *lysC*. Aspartate kinases I and III of *E. coli* are under control of the feedback inhibition by L-threonine and L-lysine, respectively, whereas aspartate kinase II is not affected by feedback inhibition [82]. Instead, aspartate kinase II of *E. coli* is regulated by L-methionine through repression of *metBL* operon [83]. Aspartate kinase of *C. glutamicum* is subjected to the feedback inhibition by both L-lysine and L-threonine [13].

Homoserine dehydrogenase and homoserine kinase are encoded in the *hom* and *thrB* genes, respectively, in *C. glutamicum*. They form an operon and its expression is repressed by L-methionine [84]. Additionally, both enzymes are feedback inhibited by L-threonine [13]. In *E. coli*, *thrA* encodes a bifunctional enzyme that

works as homoserine dehydrogenase in addition to aspartate kinase [85]. This gene forms an operon with *thrB* and *thrC*, the latter of which encodes threonine synthase. Expression of the *thrABC* operon is controlled by transcriptional attenuation by L-threonine as well as L-isoleucine [82]. Moreover, the activity of homoserine dehydrogenase and homoserine kinase is feedback inhibited [82].

For L-isoleucine production, it is advantageous to use L-lysine-producing strains to supply L-threonine because the biosynthetic pathway of L-threonine shares a significant part with that of L-lysine. Colón et al. [40] reported accumulation of 114 mM L-isoleucine using an L-lysine-producing strain *C. glutamicum* ATCC 21799 (termed as *C. lactofermentum* in the original paper) by overexpressing the wild-type *ilvA*. Morbach et al. [13, 86] used another L-lysine-producing strain of *C. glutamicum* generated by random mutagenesis. They introduced multiple copies of the feedback-resistant *hom* and *thrB* in the chromosome or replaced the chromosomal native *lysC* and *hom* genes with the feedback-resistant ones. Along with overexpression of *ilvA*, the former strain produced 96 mM L-isoleucine in the batch culture and the latter 138 mM in the fed-batch culture. It should be mentioned that overexpression of *hom* was possible only after overexpression of *ilvA* because otherwise accumulation of L-threonine and L-homoserine caused instability of the strains [42]. Yin et al. [45] identified the feedback-resistant mutants of TDH and AHAS from the L-isoleucine-producing strain *C. glutamicum* JHI3-156. When the both feedback-resistant enzymes were overexpressed in the same strain, this resultant strain produced 234 mM L-isoleucine in the fed-batch condition. A comparative proteomic study on this strain further identified up- and downregulated proteins, which were related to cell growth, L-isoleucine biosynthesis, and stress response [87].

Introduction of exogenous genes is also a useful strategy. For example, an *E. coli*-derived *ilvA* was introduced to an L-threonine-producing *B. flavum* strain, a relative bacterium to *C. glutamicum*, to yield 153 mM L-isoleucine [88]. Wang et al. [43] introduced the *E. coli* K-12-originated *thrABC* genes to *C. glutamicum*. After deletion of *alaT*, the resultant strain produced 100 mM L-isoleucine along with low concentrations of L-lysine, L-alanine, and L-valine. Guillouet et al. [89] reported the advantage of the *tdcB* gene encoding the catabolic TDH of *E. coli* over the feedback-resistant *ilvA*. The strain with the *tdcB* gene accumulated four times more L-isoleucine than the one with the *ilvA* gene and yielded 30 mM L-isoleucine in the batch culture [41].

As is the case for L-valine, export of the accumulated product is important for efficient production of L-isoleucine. Therefore, overexpression of the global regulator Lrp and the BCAA exporter BrnFE was performed in *C. glutamicum* JHI3-156 to produce 205 mM L-isoleucine [46]. Recently, Zhao et al. [47] reported increased L-isoleucine production using *C. glutamicum* IWJ001, which was identified as an L-isoleucine-producing strain by random mutagenesis. They found that the biosynthetic enzymes for L-isoleucine were significantly upregulated when the *fusA* and *frr* genes, which encode ribosome elongation factor G and ribosome recycling factor, respectively, were overexpressed. Together with overexpression of *ilvA*,

ilvB, *ilvN*, and *ppnk* (a polyphosphate/ATP-dependent NAD kinase), the resultant strain produced 217 mM L-isoleucine in 72 h of fed-batch fermentation.

Optimization of the fermentation conditions is also an issue to be addressed. Peng et al. [44] optimized DO and pH of the fermentation conditions using *C. glutamicum* JHI3-156 (termed as *B. lactofermentum* in the original paper) and finally achieved 203 mM L-isoleucine in the batch culture.

E. coli is also the target of manipulation for L-isoleucine bio-production. Hashiguchi et al. [90] strengthened the downstream reactions by introducing the plasmid with the *ilvA*, *ilvGM*, *ilvD*, and *ilvE* genes to an L-threonine-producing *E. coli* K-12 mutant, and the resultant strain produced 78 mM L-isoleucine. This strain coproduced L-valine, but they solved this problem by introducing the gene of the feedback-resistant aspartate kinase III, which successfully reduced L-valine production and increased the final concentration of L-isoleucine to 94 mM [48].

3.3 L-Leucine

In the classical examples, accumulation of L-leucine was observed in the revertants of *S. marcescens* from the L-isoleucine auxotrophic mutant, which had been generated by resistance to α-aminobutyric acid [91]. Mechanistic investigations [91, 92] revealed that the resistance to α-aminobutyric acid was acquired by derepression of both L-isoleucine/L-valine and L-leucine biosynthetic enzymes. Interestingly, the reversion from the L-isoleucine auxotrophy was not due to desensitization of the feedback inhibition of TDH, but due to that of IPMS, which allowed for overproduction of L-leucine as well as supply of 2-ketobutyrate, i.e., a precursor for biosynthesis of L-isoleucine in place of TDH.

Another L-leucine-producing mutant was obtained from the glutamic acid-producing bacterium, *B. lactofermentum* [93]. This strain was screened from an L-methionine/L-isoleucine double auxotroph of *B. lactofermentum* 2256 using 2-thiazolealanine as an amino acid metabolism competitor. The optimization of the culture conditions allowed production of 30 g/L L-leucine [94, 95]. In this strain, IPMS is both desensitized and derepressed while AHAS remained intact [56]. Therefore, another screening was performed using β-hydroxyleucine, which obtained AHAS mutants desensitized from all of the BCAAs [96]. These strains showed improved productivity of 34 g/L L-leucine. A further campaign was conducted using high concentrations of D-α-aminobutyric acid, and a mutant with higher activities of AHAS and IPMS was isolated [97]. This mutant produced more L-leucine and showed a better L-leucine/L-valine ratio than the parent strain.

Mutants of *E. coli* have been developed for L-leucine production more recently. Some 4-azaleucine-resistant strains derived from *E. coli* K-12 were desensitized to the feedback inhibition of IPMS, and the most efficient strain produced 5.2 g/L L-leucine [25]. Alternatively, the L-isoleucine/L-valine double auxotroph with mutation in *ilvE* was isolated [98]. This strain was then supplemented with the plasmid to overexpress *tyrB*, which works only on the L-leucine biosynthesis among the

BCAAs. The resultant strain produced 2.7 g/L L-leucine with no detectable L-valine or L-isoleucine. Lowering the promoter activity of the *sucAB* gene encoding α-ketoglutarate dehydrogenase is likely to reduce the carbon flux into TCA cycle and decrease consumption of acetyl-CoA [99]. When this was combined with the feedback-free IPMS and the inactivated BCAT, the resultant strain produced 11.4 g/L L-leucine.

L-Leucine-producing strains were also found from amino acid auxotrophs of *C. glutamicum*. One of the best producers was the L-phenylalanine/L-histidine double auxotroph, which accumulated 16.0 g/L L-leucine. Another example is the *S*-(2-aminoethyl)-L-cysteine-resistant mutant of *C. glutamicum* [100], though this strain was found to be unstable and generated several types of revertants during the fermentation.

Recently, a genetically defined strain which produces L-leucine was reported [49]. This strain contains three copies of the P_{tuf}-*leuA*_B018 module in the chromosome. This gene, *leuA*_B018, encodes IPMS, which is disarmed from feedback inhibition by L-leucine. Moreover, in this module, the native promoter was replaced by the strong *tuf* promoter, which is free from transcriptional attenuation. Acetyl-CoA, i.e., another substrate for the IPMS reaction, was increased by replacing the native promoter of *gltA* (encoding citrate synthase) to $P_{dapA\text{-}L1}$. The transcriptional repressor-encoding gene *ltbR* was deleted to enhance expression of the downstream genes *leuBCD* for L-leucine production. Furthermore, mutations were introduced to the regulatory site of AHAS (encoded by *ilvN*) to be desensitized from feedback inhibition and increase the carbon flux toward L-leucine production. IolT1, which is regulated by IolR, catalyzes glucose uptake in a PTS-independent manner. Therefore, the *iolR* gene was also deleted to enhance glucose uptake. This strain, *C. glutamicum* P_{tuf}-*leuA*_B018 $P_{dap\text{-}L1}$-*gltA ilvN*_fbr Δ*ltbR*::P_{tuf}-*leuA*_B018 Δ*leuA*::P_{tuf}-*leuA*_B018 Δ*iolR*, produced up to 181 mM L-leucine in the culture solution along with precipitate of L-leucine after 72 h of the fed-batch fermentation.

4 Production of Chemicals Using the BCAA Biosynthetic Pathways

Recently, the instability of oil prices and environmental concerns has been driving the microbial production of biofuels. Such research includes ethanol fermentation as well as production of higher alcohols, which are expected to be next-generation biofuels due to their high energy density [101, 102]. Isobutanol is one representative example of such alcohols.

Isobutanol can be synthesized using the L-valine biosynthetic pathway (Fig. 2). 2-Ketoisovalerate, i.e., an intermediate of the L-valine biosynthesis, is a starting material for isobutanol production. It is first converted to isobutyraldehyde by 2-ketoisovalerate decarboxylase (KIVD), and then isobutanol is formed from the aldehyde by reduction using alcohol dehydrogenase (ADH). Like L-valine

production, overexpression of the genes encoding AHAS, AHAIR, and DHAD is effective to enhance availability of 2-ketoisovalarate for isobutanol production [103]. The bacterial strains developed for isobutanol production are reported for *E. coli* [104, 105], *B. subtilis* [106], and *C. glutamicum* [107], and the yields range between 74 and 297 mM. Although less efficient, *S. cerevisiae* is also used as a host for isobutanol production. The engineered strain overexpressing the L-valine biosynthetic genes accumulated 2.4 mM isobutanol [108], and the one whose L-isoleucine biosynthesis was eliminated produced 3.0 mM [109]. One of the difficulties in microbial production of isobutanol is that this alcohol is very toxic for microbes. To address this issue, Yamamoto et al. [110] performed isobutanol production in a growth-uncoupled manner under oxygen-deprived conditions. Moreover, they continuously extracted isobutanol from the aqueous reaction phase by layering oleyl alcohol and achieved as high as 981 mM of volumetric productivity.

In a similar manner to isobutanol, 3-methyl-1-butanol and 2-methyl-1-butanol can be produced from 2-keto-4-methylvalerate and 2-keto-3-methylvalerate, i.e., intermediates for the L-leucine and L-isoleucine biosynthesis, respectively, by the catalysis of KIVD and ADH (Fig. 2) [104, 111]. Such examples include the *E. coli* strain reported by Connor and Liao [112], where accumulation of 17 mM of 3-methyl-1-butanol was achieved when expression of the L-valine and L-leucine biosynthetic genes was enhanced to increase availability of 2-keto-4-methylvalerate for 3-methyl-1-butanol production. They also performed random mutagenesis and obtained an efficient strain that was able to produce 128 mM of 3-methyl-1-butanol in the biphasic fermentation using oleyl alcohol [113]. Cann and Liao [114] engineered *E. coli* to produce 2-methyl-1-butanol. In addition to strengthening the L-isoleucine biosynthetic pathway, the flux to L-threonine was optimized, and the resultant strain produced 14 mM of 2-methyl-1-butanol. Recently, production of isobutanol (17 mM), 3-methyl-1-butanol (8.5 mM), and 2-methyl-1-butanol (12 mM) was performed by the strains engineered for each alcohol with relevant genes from *S. cerevisiae* using *C. crenatum* as a parent strain [115].

Another important chemical for which the BCAA fermentative pathway can be applied is D-pantothenate. In *C. glutamicum*, it is produced from 2-ketoisovalerate by three enzymatic reactions catalyzed by ketopantoate hydroxymethyltransferase (KPHMT), AHAIR, and pantothenate synthetase (PS) [116] (Fig. 2). Hüser et al. [117] reported a D-pantothenate-producing strain. In this strain, *ilvA* was deleted, and the transcription of *ilvE* was attenuated to decrease the carbon flux to the competing BCAA biosynthesis. Additionally, overexpression of *ilvBNCD* was performed to increase availability of 2-ketoisovalerate as well as overexpression of *panBC* to direct more 2-ketoisovalerate to the D-pantothenate biosynthetic pathway. This strain produced 8 mM of D-pantothenate.

5 Conclusion

In this chapter, the biosynthetic pathway of BCAAs and its regulatory system were described, and the microbial strains engineered for BCAA production were summarized. The classical mutagenic strains provided a heritage of information about the complicated feedback inhibition and transcriptional attenuation in the BCAA biosynthesis. They now work as a guide for the rational design of BCAA-producing strains using advanced molecular biology techniques.

Generally, genetic modifications for producing target BCAAs are divided into four stages [3]: (1) to disarm feedback inhibition by the target BCAA, (2) to remove transcriptional attenuation by the target BCAA, (3) to minimize carbon flux to the competing pathways to reduce formation of by-products, and (4) to enhance expression of genes encoding biosynthetic enzymes of a BCAA to converge carbon resources as much as possible to production of it. Additionally, engineering of the export and import systems as well as modification of the transcriptional factors or the global regulators may be beneficial to improve production of the target amino acid further.

Disarming feedback inhibition is the most important step for efficient production of BCAAs because it has a very strong inhibitory effect on the microorganism growth and therefore the fermentative production of BCAAs. For example, the minimum inhibitory concentration of L-valine to the growth of *E. coli* K-12 is reported to be as low as 2 mg/L [17]. The complicated feedback inhibition system of the BCAA biosynthetic pathways was clarified by the early-stage studies on the auxotrophic strains and the amino acid analog-resistant strains. The key players of feedback inhibition are AHAS for all BCAAs, TDH for L-isoleucine, and IPMS for L-leucine, which can be now desensitized by rational mutation.

Production of BCAAs is also affected by transcriptional attenuation of the relevant genes. Derepression of the genes coding for AHAS is a common tactic because they are related to biosynthesis of all BCAAs. Removal of transcriptional attenuation of *leuA* is also important for L-leucine production. For L-isoleucine production, increasing supply of L-threonine by relieving transcriptional attenuation of the relevant genes may be beneficial. These can be performed by replacing the native promoters with others, e.g., *tac* promoter.

Minimization of carbon flux to competing pathways is required for efficient BCAA production. This can be achieved by deleting the corresponding genes. Pyruvate, which is the common intermediate for BCAA production, is also used for production of other competing by-products such as lactate, acetate, and succinate. Therefore, the genes responsible for production of these by-products are the target of deletion to increase availability of pyruvate for the BCAA biosynthesis. Additionally, for production of one of the BCAAs, the genes specifically relevant to the other two BCAAs are to be deleted, or their expression should be suppressed. For example, the *ilvA* gene, which is involved only in the L-isoleucine biosynthesis, is the target of deletion or attenuation for L-valine production.

Finally, overexpression of the genes responsible for biosynthesis of the target amino acid enhances its productivity. In particular, overexpression of AHAS, AHAIR, and DHAD, which catalyze the common reactions for all BCAAs, is effective for their production. It is beneficial to overexpress the *ilvA* and *leuA* genes for production of L-isoleucine and L-leucine, respectively, because they code for the enzymes responsible for their specific biosynthetic pathways. In addition, enhanced expression of the exporters of BCAAs as well as their positive regulators is also beneficial.

Currently, in silico studies of carbon flux simulation are emerging as a powerful tool to design strategies to maximize efficiency of biosynthetic pathways for production of the target compound [118, 119]. Application of these techniques will become an essential part of creating more sophisticated microbial strains, which can realize our goals.

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L-Cysteine Metabolism and Fermentation in Microorganisms

Hiroshi Takagi and Iwao Ohtsu

Abstract L-Cysteine is an important amino acid both biologically and commercially. Although most amino acids are industrially produced by microbial fermentation, L-cysteine has been mainly produced by protein hydrolysis. Due to environmental and safety problems, synthetic or biotechnological products have been preferred in the market. Here, we reviewed L-cysteine metabolism, including biosynthesis, degradation, and transport, and biotechnological production (including both enzymatic and fermentation processes) of L-cysteine. The metabolic regulation of L-cysteine including novel sulfur metabolic pathways found in microorganisms is also discussed. Recent advancement in biochemical studies, genome sequencing, structural biology, and metabolome analysis has enabled us to use various approaches to achieve direct fermentation of L-cysteine from glucose. For example, worldwide companies began to supply L-cysteine and its derivatives produced by bacterial fermentation. These companies successfully optimized the original metabolism of their private strains. Basically, a combination of three factors should be required for improving L-cysteine fermentation: that is, (1) enhancing biosynthesis: overexpression of the altered *cysE* gene encoding feedback inhibition-insensitive L-serine *O*-acetyltransferase (SAT), (2) weakening degradation: knockout of the genes encoding L-cysteine desulfhydrases, and (3) exploiting export system: overexpression of the gene involved in L-cysteine transport. Moreover, we found that "thiosulfate" is much more effective sulfur source than commonly used "sulfate" for L-cysteine production in *Escherichia coli*, because thiosulfate is advantageous for saving consumption of NADPH and relating energy molecules.

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Contents

1 Introduction

L-Cysteine is the most important sulfur-containing compound because it is a sole metabolic entrance for reduced sulfur into cell metabolism in most organisms, and it is required for the biosynthesis of sulfur-containing compounds such as L-methionine, thiamine, biotin, and coenzymes A. It is also needed for Fe/S clusters of the catalytic domain of some enzymes, such as aconitase, cytochromes, and degradative L-serine dehydratase of *Escherichia coli* [1]. In addition, L-cysteine plays crucial roles in protein folding, assembly, and stability through disulfide-bond formation. Furthermore, glutaredoxin (Grx) and thioredoxin (Trx) that use an L-cysteine-containing peptide, L-glutathione, as a cofactor are involved in protecting cells under oxidative stress conditions. Recently, we have proposed that the periplasmic L-cysteine protects *E. coli* cells from hydrogen peroxide, which is produced by phagocytes, in the environment [2].

In addition to the essential function in cellular metabolism, L-cysteine is also an important amino acid in terms of its applications in the pharmaceutical (expectorant agent and freckles preventive medicine), food (dough conditioner, flavor enhancer,

and dietary supplement), and cosmetic (perm assistant and whitening agent) industries. L-Cysteine represents a global market of approximately 5,000 tons per year and is now supplied by a combination of different production technologies, which include the hydrolysis of keratin, enzymatic synthesis, and fermentation [3–5]. However, extraction methods result in low yields and cause unpleasant odors and problems of waste treatment [6]. Although L-cysteine is considered a substance that is generally recognized as safe (GRAS) by the Food and Drug Administration (FDA), synthetic or biotechnological products are presently preferable for consumers because of the bovine spongiform encephalopathy (BSE) problem in animal-origin products [7].

The metabolic pathway of L-cysteine has been well studied in *E. coli*; however, studies of other microorganisms remain limited. This chapter focuses on the recent findings on L-cysteine metabolism and advances of biotechnological production (including both enzymatic and fermentation processes) of L-cysteine. We also discussed the metabolic regulation of L-cysteine and sulfur compounds including novel sulfur metabolic pathways found in microorganisms.

2 Conventional Processes for L-Cysteine Production

The protein primary amino acids are all manufactured on an industrial scale. The production methods fall into three classes: extraction from protein hydrolysates, fermentation processes, and chemical synthesis. However, most of the amino acids can now be prepared by fermentation or synthesis, although extraction remains important for L-histidine, L-leucine, L-cysteine, and L-tyrosine [3, 8].

2.1 Extraction from Human Hairs and Animal Feathers

L-Cysteine has been obtained from acid hydrolysates of keratin, which is the key structural protein with abundant L-cysteine of human hairs and animal feathers. Prior to electrolysis, L-cysteine is extracted through a chemical process that includes treatments with activated charcoal and concentrated hydrochloric acid. This process is the simplest and easiest method for L-cysteine production, but it has drawbacks such as a low yield and unpleasant odor and also includes serious environmental and safety problems, the disposal of hydrochloric acid-containing waste liquid and the use of animal-derived raw materials, respectively.

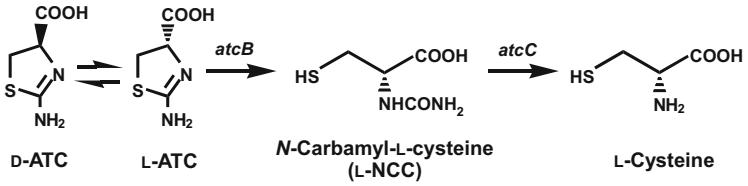

Fig. 1 A metabolic pathway of DL-2-amino-Δ^2-thiazoline-4-carboxylic acid (DL-ATC) to L-cysteine via *N*-carbamoyl-L-cysteine (L-NCC) in *Pseudomonas* species. The *atcB* and *atcC* genes encode L-ATC acid hydrolase and L-NCC amidohydrolase, respectively

2.2 Asymmetrical Hydrolysis of DL-2-Amino-Δ^2-Thiazoline-4-Carboxylic Acid

Instead of the hydrolysis of hairs, a bioconversion process for L-cysteine production has been developed [9]. Some bacteria belonging to the genus *Pseudomonas* exhibit activities involved in the asymmetric hydrolysis of DL-2-amino-Δ^2-thiazoline-4-carboxylic (DL-ATC) acid to L-cysteine [10, 11]. The conversion of DL-ATC to L-cysteine consists of the following three successive steps (Fig. 1): (1) enzymatic racemization of D-ATC to L-ATC; (2) a ring-opening reaction of L-ATC to *N*-carbamoyl-L-cysteine (L-NCC) as intermediate; and (3) hydrolysis of L-NCC to L-cysteine [12]. The improvement of this enzymatic conversion process, mainly in terms of the yield, has been reported [9]. This process has been industrialized with Ajinomoto Co., Inc.

The genes encoding L-ATC hydrolase (*atcB*) and L-NCC amidohydrolase (*atcC*) are cloned and sequenced from *Pseudomonas* sp. strain BS. The deduced amino acid sequence of the *atcC* gene product is highly homologous with other bacterial L-*N*-carbamoylases, although that of *atcB* is novel [13]. The *atcB* gene is first identified as a gene encoding an enzyme that catalyzes thiazoline ring-opening reaction and does not share high homology with other enzymes whose functions have been reported in detail. Similar results have been also reported using *Pseudomonas* sp. strain ON-4a [14].

3 L-Cysteine Biosynthesis

3.1 L-Cysteine Biosynthesis in Escherichia coli

The precursor of L-cysteine is L-serine in most bacteria and plants. L-Serine is synthesized from 3-phosphoglycerate, the glycolytic intermediate, via a three-step pathway in enteric bacteria [15]. 3-Phosphoglycerate is first oxidized to phosphohydroxypyruvate by the reaction of 3-phosphoglycerate dehydrogenase (PGDH; EC 1.1.1.95, the *serA* gene product). Subsequent transamination leads to the formation of phosphoserine, which is dephosphorylated to give L-serine

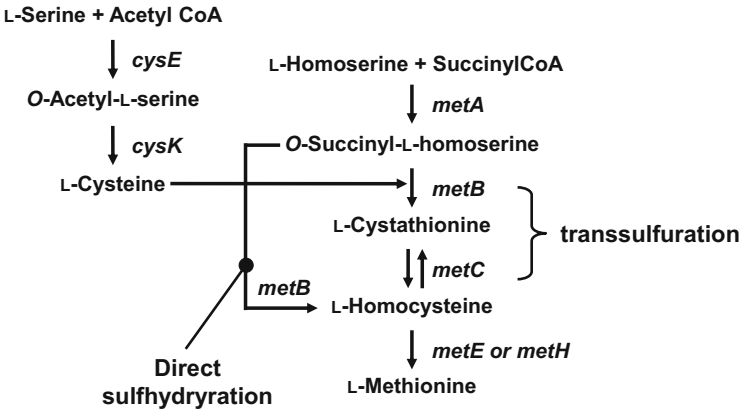

Fig. 2 Biosynthesis of L-cysteine and L-methionine in *Escherichia coli*. For L-methionine biosynthesis, *Corynebacterium glutamicum* has the direct sulfhydration pathway other than the transsulfuration pathway. The *cysE*, *cysK*, *metA*, *metB*, *metC*, *metE*, or *metH* gene encodes L-serine *O*-acetyltransferase (SAT), *O*-acetyl-L-serine sulfhydrylase-A (OASS-A), L-homoserine *O*-succinyltransferase, L-cystathionine γ-synthase/*O*-succinyl-L-homoserine sulfhydrylase, L-cystathionine β-lyase (CBL), and L-methionine synthase (vitamin B12-dependent or -independent), respectively

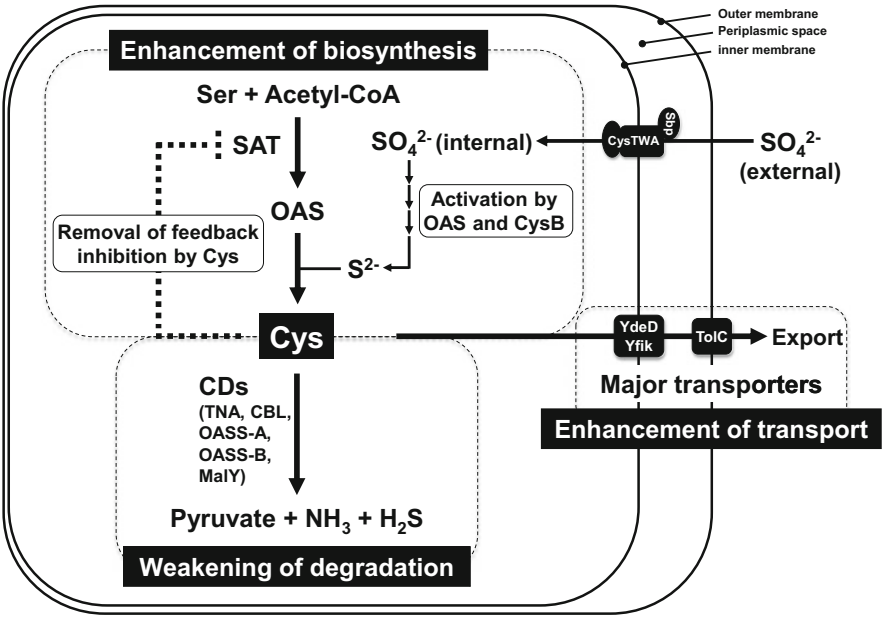

Fig. 3 Rational approach for L-cysteine fermentation by enzyme, protein, and metabolic engineering. *Ser* L-serine, *Cys* L-cysteine, *SAT* L-serine *O*-acetyltransferase, *CD* L-cysteine desulfhydrase, *TNA* tryptophanase, *CBL* L-cystathionine β-lyase, *OASS-A* OAS sulfhydrylase-A, *OASS-B* OAS sulfhydrylase-B

(catalyzed by phosphoserine aminotransferase and phosphoserine phosphatase, respectively). The activity of PGDH is inhibited by L-serine [16].

The biosynthesis of L-cysteine from L-serine in enteric bacteria such as *E. coli* (Fig. 2) and *Salmonella typhimurium* proceeds via a two-step pathway. L-Serine undergoes a substitution of its β-hydroxyl with a thiol in two steps. L-Serine *O*-acetyltransferase (SAT; EC 2.3.1.30, the *cysE* gene product) catalyzes the acetylation by acetyl-CoA of the β-hydroxyl of L-serine to give *O*-acetyl-L-serine (OAS). The second step, the α,β-elimination of acetate from OAS and the addition of H_2S to give L-cysteine, is then catalyzed by OAS sulfhydrylase-A (OASS-A; EC 4.2.99.8, the *cysK* gene product). The first reaction catalyzed by SAT is the rate-limiting step of L-cysteine biosynthesis in *E. coli* (Fig. 3). The enzyme inhibition constant (K_i) for L-cysteine of *E. coli* SAT was reported to be on the micromole order [17]. Some protein engineering approaches for obtaining feedback inhibition-insensitive SAT have been reported and are described in the section below. The SAT/OASS-A pathway was believed to be a sole L-cysteine biosynthetic pathway, and *E. coli* does not have a reverse transsulfuration pathway (Fig. 2), which converts L-cystathionine, which is the product of L-methionine metabolism, into L-cysteine. The *cysE* mutants in both *E. coli* and *S. typhimurium* showed L-cysteine auxotrophy [18].

The biosynthesis of L-cysteine in *E. coli* is regulated not only at the level of enzymatic activity (feedback inhibition) but also at that of gene expression. The cysteine regulon (known as "cys-regulon") comprises the gene for biosynthesis of L-cysteine and those for the uptake and reduction in oxidized sulfur sources, such as sulfate or thiosulfate. The expression of these genes (except for *cysE* and *cysG*) is under the control of the transcriptional activator CysB. CysB requires the inducer *N*-acetyl L-serine (which is formed from OAS by chemical reaction) for activity and sulfur limitation. Sulfide and thiosulfate act as anti-inducer for CysB. A detailed discussion of mechanism of cys-regulon can be found elsewhere [17].

3.2 L-Cysteine Biosynthesis in Other Microorganisms

In every organism, L-cysteine and L-methionine are inevitable as sulfur-containing compounds. Animals intake the amino acids as organic sulfur sources, and catabolize them to provide various cellular sulfur compounds. By contrast, most microorganisms as well as plants can assimilate environmental inorganic sulfur sources such as sulfate (SO_4^{2-}) and thiosulfate ($S_2O_3^{2-}$) into L-cysteine and the downstream metabolite L-methionine. *E. coli* has two well-known pathways for inorganic sulfur assimilation (Fig. 4) [19, 20]. One is a sulfate-utilizing "sulfate pathway," which consumes two molecules of ATP and four molecules of NADPH to synthesize one molecule of L-cysteine. Another is a thiosulfate-utilizing "thiosulfate pathway," which requires only one molecule of NADPH [19]. For incorporation of sulfur atom, both pathways use a common carbon skeleton OAS that is synthesized from L-serine and acetyl-CoA by SAT. OASS-A catalyzes the conversion of

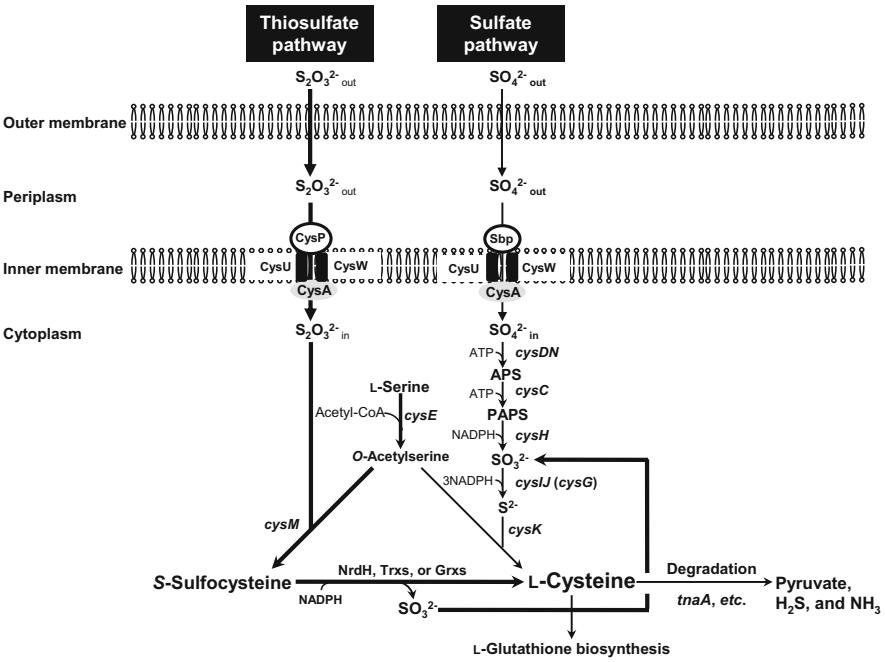

Fig. 4 Overall sulfur metabolism in *E. coli* [19]. Sulfate and thiosulfate are imported from the outside to the cytoplasm through common transporter CysTWA. *GSH* glutathione, *Trx* thioredoxin, *Grx* glutaredoxin, *APS* adenosine 5′-phosphosulfate, *PAPS* 3′-phosphoadenosine 5′-phosphosulfate

OAS and sulfide (S^{2-}) into L-cysteine. In the sulfate pathway, while in the thiosulfate pathway, OAS sulfhydrylase-B (OASS-B; EC 4.2.99.8, the *cysM* gene product) produces *S*-sulfocysteine (SSC) from OAS and thiosulfate. SSC is reductively divided into L-cysteine and sulfite (SO_3^{2-}) with NrdH and Grx1 [19]. Sulfate transport into cell is dominantly mediated by Sbp-CysTWA and thiosulfate transport is dominantly mediated by CysP-CysTWA complex on the inner membrane [20–22]. It is noteworthy that the inner-membrane channel part consisting of CysTWA is common for both uptakes (Fig. 4).

Corynebacterium glutamicum and related bacteria are Gram-positive and non-pathogenic and are known as coryneform glutamic acid-producing bacteria. Their derivatives have been used in industry for the production of various amino acids by means of fermentation. The precursor of L-cysteine is also L-serine as in the case of *E. coli*. L-Serine is synthesized from 3-phosphoglycerate, the glycolytic intermediate, via a three-step pathway same as *E. coli* [23]. In *C. glutamicum*, L-cysteine is synthesized through basically the same pathway as *E. coli*. However, *C. glutamicum* has the direct sulfhydration pathway (Fig. 2) other than the transsulfuration pathway for L-methionine biosynthesis [24]. Both SAT and OASS-A of *C. glutamicum* have been partially purified and characterized [25, 26]. The K_i for L-cysteine of *C. glutamicum* SAT is also estimated to be of

the micromole order [25]. However, detailed characterization of purified *C. glutamicum* SAT has not yet been reported. Protein engineering on *C. glutamicum* SAT, such as construction of the feedback inhibition-insensitive mutant enzyme, also remains to be investigated.

Some microorganisms, such as *Saccharomyces cerevisiae*, *Lactococcus lactis* [27], and *Pseudomonas putida* [28], have been reported to synthesize L-cysteine from L-methionine through the reverse transsulfuration pathway (Fig. 2). With regard to *S. cerevisiae*, previous studies concluded that detectable SAT and OASS-A do not constitute an L-cysteine biosynthetic pathway and that L-cysteine is exclusively synthesized via cystathionine by L-cystathionine β-synthase (CBS; EC 4.2.1.22) and L-cystathionine γ-lyase (CGL; EC 4.4.1.1) [29, 30]. Wheeler et al. [31] reported that pathogenic bacteria, namely, the *Mycobacterium tuberculosis* complex, have a reverse transsulfuration pathway and can synthesize cysteine from methionine. However, *C. glutamicum*, which is taxonomically closely related to *M. tuberculosis*, cannot synthesize L-cysteine from L-methionine [25]. L-Cysteine production by *C. glutamicum* has been reported, although the productivity was relatively low (290 mg/L) compared with the productivity of that produced by *E. coli* [32].

Some archaea have been reported to synthesize L-cysteine from L-methionine through reverse transsulfuration from L-homocysteine to L-cysteine [33]. On the other hand, several archaea are considered to have genes encoding SAT and OASS [34]. In an extremely thermophilic bacterium, *Thermus thermophilus*, L-cysteine is synthesized with OAS and sulfide by the catalysis of OASS-A [35]. This organism catalyzes the reactions of transsulfuration from L-cysteine to L-homocysteine when ammonium sulfate is used as the sulfur source, but is unable to catalyze reverse transsulfuration because of the absence of CBS [35]. It was suggested that OASS-A purified from *T. thermophilus* HB8 is responsible for the synthesis of L-cysteine in this organism cultured with either sulfate or L-methionine given as the sole sulfur source [36]. The *sat1* gene encoding SAT of *T. thermophilus* HB8 was cloned and overexpressed in *E. coli* cells, based on the genome sequence [37]. The predicted amino acid sequence was homologous to those of the *O*-acetyltransferase members. The recombinant enzyme was active over a wide range of temperatures, with maximum activity at around 75°C. Interestingly, the enzyme was highly activated by Co^{2+} or Ni^{2+}, and contained Zn^{2+} and Fe^{2+}, indicating that the *T. thermophilus* SAT is a novel type of enzyme different from this protein family [37].

4 L-Cysteine Degradation

In *E. coli*, L-cysteine degradation is catalyzed mainly by L-cysteine desulfhydrase (CD) [38]. Enzymes having CD activity in *E. coli* have been investigated in considerable detail (Fig. 3). Awano et al. [39] reported that tryptophanase (TNA; EC 4.1.99.1, the *tnaA* gene product) and L-cystathionine β-lyase (CBL; EC 4.4.1.8, the *metC* gene product) catalyzed CD reaction and acted on L-cysteine degradation

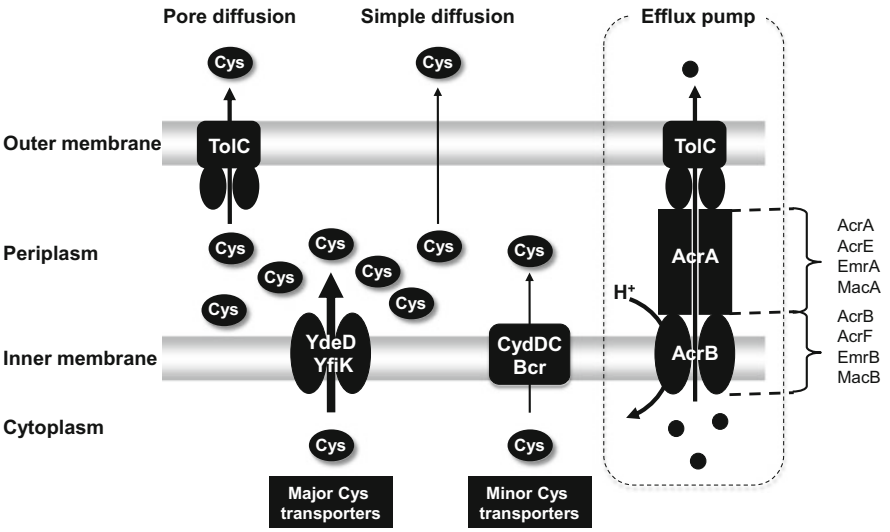

Fig. 5 L-Cysteine export systems in *E. coli*. It is known that the outer membrane channel TolC forms various types of tripartite efflux pump in Gram-negative bacteria (*dashed box*), and serves as an essential element of some mechanisms responsible for tolerance to various compounds, including hydrophobic antibiotics and organic solvents [43]. TolC interacts with an inner-membrane transporter, AcrB, which provides export energy, and a protein of the adaptor family, AcrA, which brings TolC and the inner-membrane transporter together. In addition, the TolC channel also exports L-cysteine from the periplasm to outside the cell [44]. In contrast, both major (YdeD and YfiK) and minor (CydDC and Bcr) L-cysteine transporters once export L-cysteine from the cytoplasm to the periplasm, and then the periplasmic L-cysteine diffuses to outside the cell through TolC [44]

in *E. coli* by analyses with CD activity staining and gene disruption. Subsequently, using the plasmid gene library of *E. coli*, which contains 4,388 kinds of open reading frame, three additional proteins having CD activity were identified as OASS-A, OASS-B, and MalY protein in *E. coli* [40]. These five CD proteins (TNA, CBL, OASS-A, OASS-B, and MalY) involved in L-cysteine degradation are all pyridoxal 5′-phosphate (PLP)-dependent enzymes. Therefore, the CD activity of these enzymes may be only a side reaction that is often observed in PLP-dependent enzymes due to the chemistry of PLP. Growth phenotype and transcriptional analyses suggest that the CD activity of TNA contributes primarily to L-cysteine degradation in vivo. In fact, TNA, which primarily degrades L-tryptophan to indole, pyruvate, and ammonia, is induced by the addition of L-cysteine to the culture medium, suggesting that a novel transcriptional regulation system is involved in the gene expression. However, it appears that unidentified proteins with CD activity, which may be induced by L-cysteine, are still present in *E. coli*. It is possible that one gene disruption would affect the expression of other CD proteins and the whole metabolic profile in *E. coli* [40].

In *C. glutamicum*, only one CD enzyme has been purified and characterized [32]. The enzyme was identified as the *aecD* gene product, a C-S lyase with a β-elimination activity [41]. This enzyme is also annotated as CBL [42]. Thus, the CD activity of this enzyme may be a side reaction of CBL as in the case of *E. coli*. However, other enzymes having CD activity in *C. glutamicum* remain to be clarified. Disruption of the *aecD* gene was effective for L-cysteine production by *C. glutamicum*, as in the case of *E. coli* [32].

5 L-Cysteine Transport

Export systems for L-cysteine in *E. coli* were also well studied. Previously, two transporter proteins, YdeD and YfiK, have been identified as L-cysteine exporters in *E. coli* (Fig. 5) [45, 46]. Both YdeD and YfiK are membrane-integral proteins and belong to different protein families. Overexpression of either YdeD or YfiK led to parallel secretion of OAS and L-cysteine by *E. coli* cells [47]. L-Cysteine is also exported from the *E. coli* cytoplasm to the periplasm by CydDC, which is an ATP-binding cassette (ABC)-type transporter required for cytochrome assembly [48]. CydDC overexpression conferred resistance to high extracellular L-cysteine concentrations. However, CydDC was shown to have higher transport activity with L-glutathione than with L-cysteine as the substrate [49].

In addition to these transporters, there are 37 open reading frames assumed to be drug transporter genes on the basis of sequence similarities in *E. coli*, although the transport abilities of most of them have not yet been established [50]. Five families of drug extrusion translocases have been previously identified based on sequence similarity, including the major facilitator (MF) family, the small multidrug resistance (SMR) family, the resistance nodulation cell division (RND) family, the ABC family, and the multidrug and toxic compound extrusion (MATE) family. Among the 33 putative drug transporter genes tested, Yamada et al. [51] reported that the multidrug transporter Bcr in the MF family is involved in the export in *E. coli* cells (Fig. 5). Amino acid transport assays showed that Bcr overexpression conferring bicyclomycin and tetracycline resistance specifically promotes L-cysteine export driven by energy derived from the proton gradient.

Recently, the *tolC* gene encoding the outer membrane channel TolC was identified as a novel gene involved in L-cysteine export using a systematic and comprehensive collection of gene-disrupted *E. coli* K-12 mutants (the Keio collection) (Fig. 5) [44]. Gene expression analysis revealed that the *tolC* gene is essential for L-cysteine tolerance, which is not mediated by TolC-dependent drug efflux systems such as AcrA and AcrB, in *E. coli* cells. It also appears that other outer membrane porins including OmpA and OmpF do not participate in TolC-dependent L-cysteine tolerance.

6 Direct Fermentation of L-Cysteine from Glucose

6.1 Enhancement of L-Cysteine Biosynthesis

As described above, the reaction catalyzed by SAT is the rate-limiting step of L-cysteine biosynthesis in many microorganisms. Due to feedback inhibition by L-cysteine of SAT, a high level of production of L-cysteine from glucose has not yet been successfully achieved in microorganisms. Thus, for the fermentative production of L-cysteine, it is necessary to use feedback inhibition-insensitive SATs.

There have been two approaches to obtaining SAT that is less sensitive to feedback inhibition: (1) the engineering of SAT from *E. coli* through site-directed or random mutagenesis [52, 53] and (2) the use of the natural SAT, which is desensitized to feedback inhibition, from higher plants [54, 55].

Denk and Böck [56] first reported that a small amount of L-cysteine (30 mg/L) was secreted by a revertant from an L-cysteine auxotroph of *E. coli*. In this revertant, SAT was less sensitive to feedback inhibition, and the Met residue at position 256 (Met256) in SAT was replaced with Ile. Because this residue was supposedly part of the allosteric site for L-cysteine binding, the mutant *cysE* genes encoding SAT variants, which were genetically desensitized to feedback inhibition, were constructed by replacing Met256 with 19 other amino acid residues using site-directed mutagenesis [52]. It was found that in the L-cysteine non-utilizing *E. coli* cells expressing the mutant *cysE* gene, there was a marked production of L-cysteine and that stable expression of feedback inhibition-insensitive SAT was necessary for the overproduction of L-cysteine (at a maximum of 790 mg/L for the Met256Ala variant). PCR random mutagenesis was introduced into the *cysE* gene to isolate the variant SATs that cause overproduction of L-cysteine, due to a much lower level of feedback inhibition [53]. The production test of L-cysteine and the enzymatic analysis of SAT variants suggested that the C-terminal region of SAT plays an important role in the desensitization to feedback inhibition and in the high level of production of L-cysteine (at a maximum of 990 mg/L for the Met201Arg variant).

The alternative way to obtain feedback inhibition-insensitive SAT is to use the natural SAT resources from higher plants. In *Arabidopsis thaliana*, three cDNA clones encoding organelle-localized SAT have been isolated: SAT-c (a cytosolic isoform), SAT-m (a mitochondrial isoform), and SAT-p (a plastidic isoform). Noji et al. [57] found that SAT-m and SAT-p were feedback inhibition-insensitive isozymes (not inhibited in the presence of up to 100 μM L-cysteine), whereas the concentrations for 50% inhibition (IC_{50}) of SAT-c and *E. coli* SAT under the standard assay condition were 1.8 and 6.0 μM, respectively. As expected, the expression of two cDNAs encoding SAT-m and SAT-p in the L-cysteine non-utilizing *E. coli* cells significantly increased the productivity of L-cysteine (1,590 and 1,660 mg/L, respectively) [54]. The application of cDNAs encoding L-cysteine-insensitive SAT from *Nicotiana tabacum* has been also reported [55].

However, these SATs showed significant decreases in enzymatic activity relative to the *E. coli* wild-type enzyme. Further improvements in L-cysteine production

are therefore expected to use an engineered SAT, which shows a higher level of feedback desensitization and a higher catalytic activity. Previously, the three-dimensional structure of SAT from *E. coli* with its inhibitor L-cysteine was determined by X-ray crystallography at a 2.2-Å resolution [58]. The crystal structure and the reaction mechanism of SAT from *E. coli* have shown that SAT is a trimeric structure, which is likely to interact with another SAT trimer at N-terminal ends, and that the substrate L-serine and the inhibitor L-cysteine bind to the identical region in SAT. To decrease the affinity for L-cysteine alone, Kai et al. [59] built the first structure model of L-serine-binding SAT on the basis of the crystal structure with bound L-cysteine and compared these two structures (L-serine- and L-cysteine-binding SAT). The comparison showed that the Cα of Asp92 underwent a substantial positional change upon the replacement of L-cysteine by L-serine. Then, various amino acid substitutions at positions 89–96 around Asp92 were introduced by randomized, fragment-directed mutagenesis to change the position of the Asp92. As a result, SAT variants, which have both extreme insensitivity to inhibition by L-cysteine ($IC_{50} = 1{,}100$ μM) and extremely high enzymatic activities, were successfully obtained [59]. Such structural information is a powerful tool for breeding an L-cysteine overproducer.

In addition, further improvement in L-cysteine productivity might be possible using SAT with high stability. Thus, a thermostable SAT of *T. thermophilus* HB8 [37] should be engineered to desensitize it to feedback inhibition based on our previous reports [52–54].

6.2 Weakening of L-Cysteine Degradation

A decrease in the degradation activity for the desired amino acid is also a promising approach to further improving amino acid production by means of fermentation. The gene disruption for each protein (TNA, CBL, OSAA-A, OASS-B, and MalY) was significantly effective for overproduction of L-cysteine. The single or quintet gene mutants transformed with the plasmid containing the *cysE* gene for feedback-insensitive SAT exhibited higher L-cysteine productivity than the wild-type strain [39, 40]. The amounts of L-cysteine produced after 72 h of cultivation increased by a factor of 1.8–2.3. However, L-cysteine productivity decreased significantly after 96 h of cultivation in all the strains, probably because of the remaining CD enzyme (s). It is also noteworthy that the quintet gene-disrupted strain (Δ*tnaA*, Δ*metC*, Δ*cysK*, Δ*cysM*, and Δ*malY*) in the presence of L-cysteine showed higher CD activity than that observed in the absence of L-cysteine. It appears that other CDs, in addition to the five proteins identified, could be induced by L-cysteine in *E. coli* [40].

6.3 Enhancement of L-Cysteine Transport

For effective amino acid production, not only the biosynthetic pathway of target amino acid but also its efflux from the cell is important. For example, L-lysine exporter, LysE, is reported to be essential for L-lysine production by *C. glutamicum*. In the absence of LysE, L-lysine can reach an intracellular concentration of more than 1,100 mM, which prevents cell growth [60]. High concentrations of L-cysteine have been also reported to be inhibitory or even toxic to *E. coli* cells [61, 62]. To produce such a toxic compound as L-cysteine by fermentation, the use of an L-cysteine efflux system could be promising, as in the case of other amino acids [63]. Yamada et al. [51] reported that the multidrug transporter Bcr is involved in overproduction of L-cysteine in *E. coli*. When the *bcr* gene was overexpressed in *tnaA*-disrupted cells expressing the *cysE* gene for feedback inhibition-insensitive SAT, the L-cysteine level was approximately fivefold higher than that of the cells harboring the vector only. It was also concluded that the outer membrane TolC plays an important role in L-cysteine tolerance probably due to its export ability and that TolC overexpression is effective for L-cysteine production in *E. coli* [44]. To improve L-cysteine production, plasmid pDES carrying the wild-type *ydeD* gene and mutant *cysE* and *serA* gene, which encodes feedback inhibition-insensitive SAT and PGDH, respectively (personal communication), was introduced into *tnaA*-disrupted cells. The transformant cells produced approximately 20% higher amount of L-cysteine than cells carrying the vector only.

Although there is insufficient information for achieving L-cysteine fermentation, two worldwide companies (Wacker Chemie AG, Germany and Ajinomoto Co., Inc., Japan) independently began to supply L-cysteine and its derivatives produced by a microbial fermentation process developed in 2001 and 2014, respectively (http://kyowa-usa.com/news/2001/06-25, http://www.ajinomoto-usa.com/Pr/Pdf/Cysteine_Derivatives.pdf). These companies could successfully optimize the original metabolism of their private bacterial strains. In particular, Wacker Chemie AG announced that a large quantity of L-cysteine was secreted into the culture medium from bacterial cells, as 90% of pure L-cysteine ends up in the final product, which fulfills the quality standards for foods and pharmaceutical industries (Wacker [64]). Of all the L-cysteine manufactured worldwide in 2004, only 12% were fermentation-origin products. Annual growth rate for the market share for microbial L-cysteine was estimated at 10% [5].

7 Novel Pathways of L-Cysteine Metabolism in *Escherichia coli*

7.1 *Thioredoxin/Glutaredoxin-Mediated L-Cysteine Biosynthesis from* S-*Sulfocysteine*

E. coli has two enzymes which assimilate inorganic sulfur sources, OASS-A (CysK) and OASS-B (CysM) (Fig. 5). While the former enzyme utilizes sulfide (S^{2-}) as a sulfur donor, the latter enzyme uses thiosulfate ($S_2O_3^{2-}$). CysK synthesizes L-cysteine from OAS and sulfide, but the CysM protein differs in that it can also utilize thiosulfate instead of sulfide. The product formed by the CysM activity, SSC, is converted into L-cysteine and sulfite by an uncharacterized reaction [65]. The *E. coli* sulfite reductase consists of the alpha subunit protein (the *cysJ* gene product) and the beta subunit protein (the *cysI* gene product). The "sulfate pathway" spends two molecules of ATP and four molecules of NADPH as a reducing power to make one molecule of L-cysteine from a sulfate and OAS molecule. On the other hand, the thiosulfate pathway has the advantage that two molecules of L-cysteine can synthesize from a thiosulfate molecule by consuming only four molecules of NADPH. As thioredoxins (Trx1 and Trx2) and glutaredoxins (Grx1, Grx2, Grx3, Grx4, and NrdH) are known as reductases of peptidyl disulfides [66], overexpression of these reductases might contribute to improving L-cysteine production by accelerating the reduction of SSC.

To test whether these enzymes catalyze the reduction of SSC to L-cysteine, all His-tagged recombinant enzymes, except for Grx4, efficiently convert SSC into L-cysteine in vitro [19]. Overexpression of Grx1 and NrdH enhanced a 15%–40% increase in L-cysteine production in *E. coli*. On the other hand, disruption of *cysM* cancelled the effect caused by the overexpression of Grx1 and NrdH, suggesting that its improvement was due to the efficient reduction of SSC. Moreover, L-cysteine production in knockout mutants of the sulfite reductase genes (Δ*cysI* and Δ*cysJ*) and the OASS-A gene (Δ*cysK*) each decreased to about 50% of that in the wild-type strain. Interestingly, there was no significant difference in L-cysteine production between wild-type strain and gene deletion mutant of the upstream pathway of sulfite (Δ*cysC* or Δ*cysH*), indicating that sulfite generated from the SSC reduction is available as the sulfur source to produce additional L-cysteine. It was finally found that the *E. coli* L-cysteine producer that co-overexpresses Grx (NrdH), sulfite reductase (CysI), and OASS-A (CysK) exhibited the highest amount of L-cysteine produced per cell, proposing that the enhancement of Trx/Grx-mediated L-cysteine synthesis from SSC is a novel method for improvement of L-cysteine production [19].

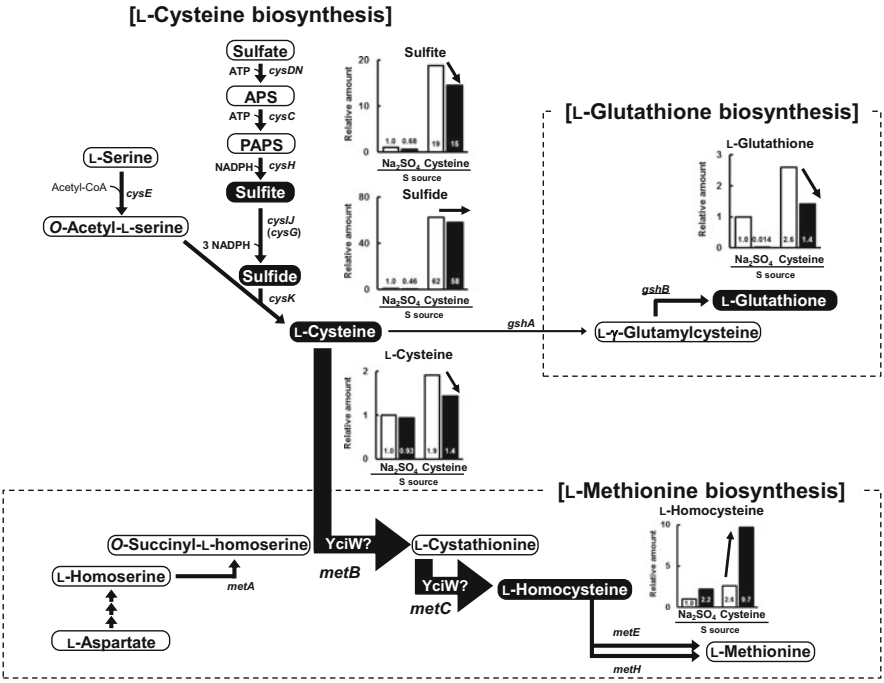

Fig. 6 The possible function of YciW in *E. coli* by sulfur metabolome [71]. YciW seems to metabolize L-cysteine and *O*-succinyl-L-homoserine to L-homocysteine via L-cystathionine. The graphs of the measured sulfur compound contents are shown in the metabolic pathway map. The metabolites represented by inverted style are the targets of LC-MS/MS analysis. The content of each metabolite in wild-type cells carrying the empty vector and overexpressing the *yciW* gene is shown by open and closed bar, respectively

7.2 *Involvement of the* yciW *Gene in* L*-Cysteine and* L*-Methionine Metabolism*

Transcription of the sulfate pathway genes is known to be upregulated during cellular L-cysteine shortage by a transcriptional activator CysB (LysR family) [67]. Using in silico analysis, which are MEME and FIMO program (http://meme.nbcr.net/meme/), the *yciW* gene of *E. coli* was identified as a novel L-cysteine regulon that may be regulated by the transcriptional activator CysB for sulfur metabolic genes [68]. To discover a novel CysB-binding motif on the *E. coli* genome, the CysB-binding motif was first predicted from the upstream sequences (300 bp from start codon) of already-known genes for CysB binding (*cysD*, *cysJ*, *cysK*, and *cysP*) [69, 70] by the MEME program (Fig. 6). The isolated motif was consistent with the already-known CysB-binding motif. Subsequently, the predicted motif (42 bp) was searched for the *E. coli* W3110 genome (accession No.: NC_007779.1) using the FIMO program. It was revealed the most plausible

sequence for the CysB-binding motif in the upstream of the *yciW* gene, which is suggested to be a new member of the CysB regulons.

Interestingly, *yciW* conferred tolerance to L-cysteine on *E. coli* cells, suggesting that the *yciW* gene product (YciW) converts L-cysteine to L-methionine or L-glutathione. Actually, the intracellular contents of all sulfur metabolites relatively increased regardless of *yciW* overexpression, suggesting that excess L-cysteine was degraded by enzyme such as L-cysteine desulfhydrases to protect *E. coli* cells from L-cysteine toxicity. After 72 h of cultivation, Δ*yciW* strain carrying pDES produced about 2.4-fold higher amount of L-cysteine per cell than wild-type strain, when sulfate was used as sulfur source. Also, when thiosulfate was used, disruption of *yciW* significantly increased L-cysteine production of *E. coli*. In contrast, overexpression of *yciW* significantly decreased total amount of L-cysteine in wild-type cells in the presence of sulfate and thiosulfate. From these results, disruption of *yciW*, one of the CysB regulons, enhances L-cysteine production. In silico gene screening is an effective method for improvement of L-cysteine production [68].

Kawano et al. [71] also analyzed a sulfur index of *E. coli* cells using LC-MS/MS combined with thiol-specific derivatization by monobromobimane. The obtained sulfur index was then applied to evaluate the L-cysteine producer. *E. coli* cells overexpressing *yciW* accumulated L-homocysteine, suggesting that YciW is involved in the biosynthesis of L-methionine rather than L-glutathione. From the metabolome analysis of sulfur compounds in *E. coli*, YciW seems to metabolize L-cysteine and *O*-succinyl-L-homoserine to L-homocysteine via L-cystathionine (Fig. 6).

8 L-Cysteine/L-Cystine Shuttle System

Excessive reactive oxygen species (ROS) are cytotoxic molecules, which result in the oxidation of DNA, proteins, and cellular membrane lipids [72]. Inside the cell, it generates from their respiration chain and various redox reactions, which results in the exchange of one or two electrons to molecular oxygen [73]. Most organisms acquire various ROS-scavenging strategies for their survival and conservation of the species. For example, *E. coli* possesses ROS-scavenging enzymes such as superoxide dismutase ($2O_2^- + 2H^+ \rightarrow O_2 + H_2O_2$), catalase ($2H_2O_2 \rightarrow O_2 + 2H_2O$), and peroxidase ($ROOR' + 2e^- + 2H^+ \rightarrow ROH + R'OH$). Superoxide dismutases exist both in the cytoplasm (SodA and SodB) and the periplasm (SodC), whereas catalase is present only in the cytoplasm (KatE) but not in the periplasm. Unlike the cytoplasm, the mechanism underlying the process for scavenging periplasmic H_2O_2 generated from the SodC reaction has remained unclear [44].

Recently, the *E. coli* mutants lacking the L-cysteine exporter YdeD or the L-cystine-binding protein FliY increased sensitivity to H_2O_2 [2]. It was also shown that FliY is involved in the uptake of L-cystine from the periplasm to the cytoplasm. Moreover, the expressions of the *ydeD* and *fliY* genes were dramatically

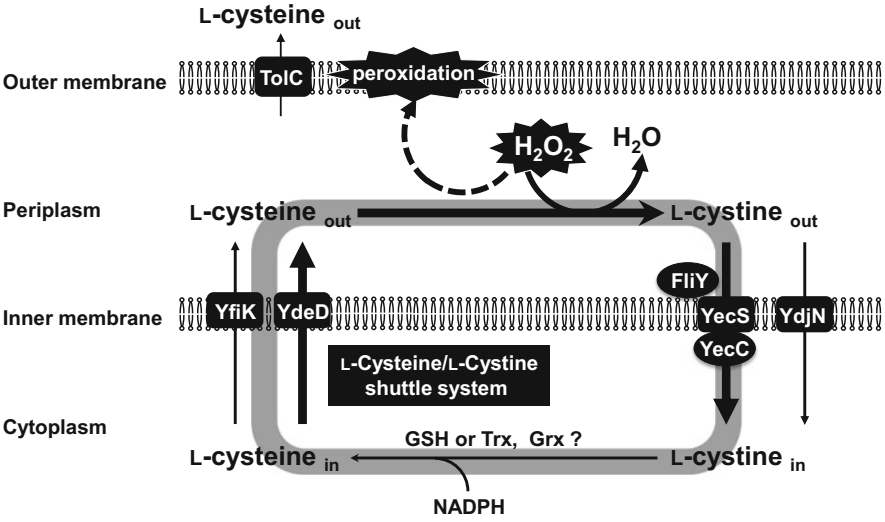

Fig. 7 Proposed L-cysteine/L-cystine shuttle system [2, 74]. The L-cysteine/L-cystine shuttle system participates in preventing membrane lipids or controlling the level of membrane peroxidation products

increased upon treatment of the cells with H_2O_2 [2]. From these findings, it was proposed that *E. coli* removes the periplasmic H_2O_2 using L-cysteine supplied to the periplasm from the cytoplasm by "L-cysteine/L-cystine shuttle system" (Fig. 7) [2]. In this system, however, it remains questionable: (1) whether Δ*ydeD* mutant cells actually accumulate H_2O_2 in the periplasm, (2) what is the FliY-dependent L-cystine importer, and (3) whether the L-cystine importer cooperatively operates with YdeD. In addition to them, the question arises as to whether endogenous L-cysteine de novo synthesized from inorganic sulfur source serves the function of L-cysteine/L-cystine shuttle system.

Thus, not only L-cysteine exporter such as YdeD but also an L-cystine importer such as the FliY-dependent protein is critical for protecting cells from oxidative stress into the periplasm due to quality control of the membrane. Ohtsu et al. [74] recently identified two different L-cystine importers, YecS and YdjN, which indeed function against H_2O_2 stress in *E. coli* (Fig. 7). These L-cystine importers cooperatively function with the L-cysteine exporter YdeD to scavenge the periplasmic H_2O_2. Furthermore, endogenous L-cysteine de novo synthesized from inorganic sulfur source was shown to eliminate the periplasmic H_2O_2. Thus, it was proposed that H_2O_2-inducible L-cysteine/L-cystine shuttle system has an important role for quality control of the plasma membrane in *E. coli*. In addition, the *yfiK* gene encoding another major L-cysteine transporter, YfiK, is induced by H_2O_2, although both the genes encoding Bcr and CydDC are not upregulated.

Table 1 L-Cysteine fermentative production in *E. coli*

Improvement of L-cysteine production	*E. coli* host strain; genotype	L-Cysteine productivity (mg/L)	Reference
Enhancing biosynthesis	JM240; *cysE*^{M256I}	ca. 30	[56]
Enhancing excretion	W3110; pYdeD	ca. 70	[45]
Enhancing excretion	W3110; pYfiK	ca. 150	[46]
Enhancing biosynthesis	JM39; *cysE51* + p*cysE*^{M256X}	130–280	[52]
Weakening degradation	JM39-8; *cysE51*	260–490	[53]
Enhancing biosynthesis/Weakening degradation	JM39; *cysE51* + p*cysE*^{random}	480–670	[53]
Enhancing biosynthesis and excretion/Weakening degradation	MG1655; Δ*tnaA* + pDES	890–1,030	[44]
Enhancing biosynthesis and excretion/Weakening degradation	MG1655; Δ*tnaA* + pDES + pTolC	1,050–1,200	
Enhancing biosynthesis and excretion	BW25113; pDES	1,200–1,230	[19]
Enhancing biosynthesis and excretion/Weakening degradation	BW25113; Δ*yciW* + pDES	1,480–1,720	[68]

Strain JM39 is a cysteine auxotroph. Strain JM39-8 has only 10% of the cysteine desulfhydrase activity of JM39 [52]
Plasmid pEDS carries the altered *serA* (Thr410 to Stop codon) and *cysE* (Thr167Ala) genes and the wild-type *ydeD* gene under the control of the *ompA* gene promoter
Plasmid p*cysE*^{M256X} were constructed by site-directed mutagenesis to replace Met256 of serine *O*-acetyltransferase with 19 other anino acids (X) [52]
Plsmid p*cysE*^{random} were constructed by PCR random mutagenesis to isolate serine *O*-acetyltransferase variants [53]

9 Conclusions and Future Perspective

Recent advancement in biochemical studies, genome sequencing, structural biology, and metabolome analysis has enabled us to use various approaches to achieve direct fermentation of L-cysteine from glucose. Table 1 summarizes the improved productivity of L-cysteine fermentation by *E. coli* cells. In addition to a feedback inhibition-insensitive SAT, low activity of L-cysteine degradation and high activity of L-cysteine-export in the host cells are very important for efficient L-cysteine fermentation. In conclusion, we believe that a combination of the three abovementioned factors should be required for achieving L-cysteine fermentation: that is, (1) overexpression of the feedback inhibition-insensitive SAT gene, (2) knockout of the genes encoding CDs, and (3) overexpression of the gene involved in L-cysteine export (Fig. 3).

Recently, we found that *S. cerevisiae* utilizes thiosulfate as a sole sulfur source. The energetically favored thiosulfate rather than sulfate as sulfur sources is also more effective for improving growth and ethanol-production rate in yeast cells due to high levels of intracellular NADPH during thiosulfate utilization [75].

Thiosulfate is an advantageous sulfur source than sulfate for saving consumption of NADPH and relating energy molecules. This is probably attributed to less NADPH consumption for L-cysteine/L-methionine biosynthesis in thiosulfate compared to sulfate. Because reducing balance of one of two sulfur atoms of thiosulfate is equal to that of sulfide, thiosulfate is much more effective sulfur source than sulfate for L-cysteine fermentative production in *E. coli* [19, 68, 71].

We thus propose that thiosulfate compared to commonly used sulfate is effective sulfur source for biotechnological production of useful compounds other than ethanol production in *S. cerevisiae*. According to this idea, we are going to try production of other useful products using thiosulfate as sulfur source, and also to elucidate molecular basis of the assimilation pathway and responsible enzymes [75].

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L-Methionine Production

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Abstract L-Methionine has been used in various industrial applications such as the production of feed and food additives and has been used as a raw material for medical supplies and drugs. It functions not only as an essential amino acid but also as a physiological effector, for example, by inhibiting fat accumulation and enhancing immune response. Producing methionine from fermentation is beneficial in that microorganisms can produce L-methionine selectively using eco-sustainable processes. Nevertheless, the fermentative method has not been used on an industrial scale because it is not competitive economically compared with chemical synthesis methods. Presented are efforts to develop suitable strains, engineered enzymes, and alternative process of producing L-methionine that overcomes problems of conventional fermentation methods. One of the alternative processes is a two-step process in which the L-methionine precursor is produced by fermentation and then converted to L-methionine by enzymes. Directed efforts toward strain development and enhanced enzyme engineering will advance industrial production of L-methionine based on fermentation.

Keywords Enzyme engineering, Feed additives, Methionine, Microbial fermentation, Strain development

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Contents

1 Introduction

Methionine is one of two sulfur-containing amino acids along with cysteine. As an essential amino acid, intake of methionine from food is required for humans and other animals [1]. Methionine is used primarily as a building block of proteins in the body. Moreover, it plays a critical role in diverse methyl-transferring reactions as a major source of methyl groups, particularly in the form of *S*-adenosylmethionine (SAM). Methionine, like lysine, is a highly important feed additive and can be added to poultry feed as the first limiting amino acid [2]. Recently, demands for methionine in aqua feed have increased because it can be used as an alternative to fish meal [3]. To use methionine for food and pharmaceuticals, DL-methionine needs to be converted to L-methionine using acetylation and enzymatic processes [4, 5]. However, the market size for methionine for food and pharmaceuticals is significantly smaller than for feed additives.

Industrial production of methionine was first done by Degussa AG (Evonik) in the 1950s. The amount of DL-methionine, produced chemically, reached to around 850,000 tons annually in 2013 [6], including methionine hydroxy analogue. DL-methionine is synthesized using acrolein, methanethiol, hydrogen cyanide, and other chemicals [7], and it is crucial to dispose hazardous intermediates appropriately to prevent any harmful impacts to the environment. Also decreasing fossil resources should be considered.

Efforts to produce methionine using microbial fermentation have been ongoing for the last three decades [8–10]. From the 1990s to the 2000s, research efforts have focused on developing suitable strains through classical mutagenesis [11, 12]. Since the 2000s, extensive metabolic engineering [13] has been conducted on major hosts such as *Corynebacterium glutamicum* and *Escherichia coli*. This review will discuss the current status of fermentative synthesis of methionine. Then, characteristics of key enzymes and achievements in strain development for industrial production of methionine will be discussed.

2 Methionine Biosynthetic Pathways

2.1 *The Methionine Biosynthetic Pathway in C. glutamicum and E. coli*

Methionine, lysine, and threonine are biosynthesized using aspartate as a precursor in *C. glutamicum* and *E. coli*. However, there are several differences in biosynthetic intermediates and regulatory mechanisms for methionine synthesis depending on the host microorganisms [9].

C. glutamicum converts aspartate to homoserine using aspartokinase (*lysC*; henceforth, the gene name is indicated in parenthesis for the protein it is encoding in a given microorganism) [14], aspartate-semialdehyde dehydrogenase (*asd*) [15], and homoserine dehydrogenase (*hom*) [16] as shown in Fig. 1. To synthesize methionine, acetylhomoserine transferase (*metX*) [17] activates homoserine to O-acetyl homoserine (OAH) with acetyl-CoA as a donor of the acetyl group. Next, for assimilation of sulfur, *C. glutamicum* has two alternative pathways. When using cysteine as a sulfur source, the synthesis pathway uses cystathionine-γ-synthase (*metB*) [18] and cystathionine-β-lyase (*metC*) [19], thus producing L-homocysteine

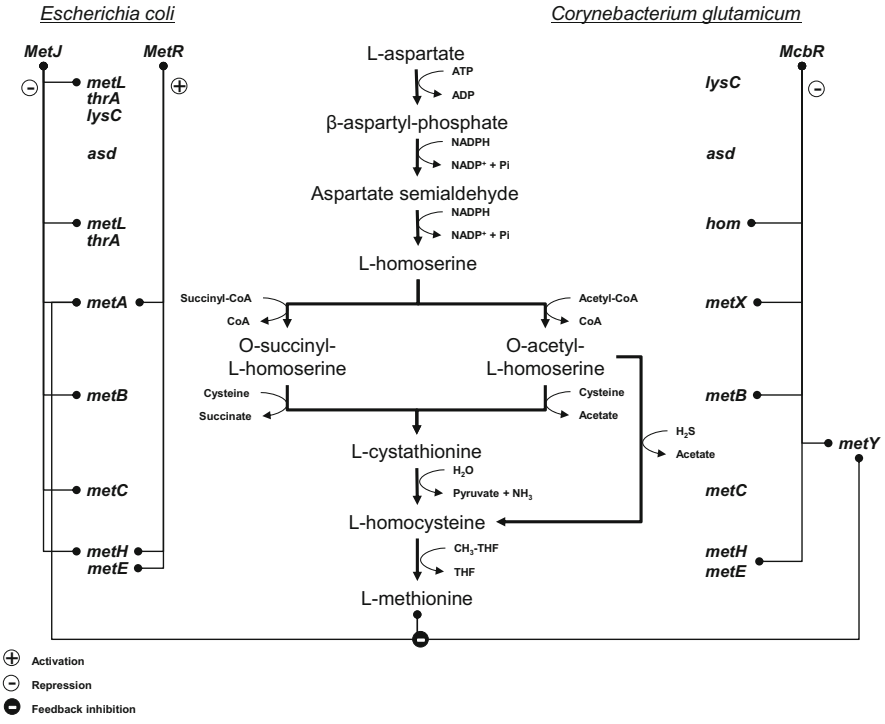

Fig. 1 Methionine biosynthesis pathway

from OAH. The other pathway uses O-acetyl homoserine sulfhydrylase (*metY*) [20, 21] and produces L-homocysteine with hydrogen sulfide as a sulfur source. The last step of methionine biosynthesis is completed by vitamin B_{12}-dependent methionine synthase (*metH*) or vitamin B_{12}-independent methionine synthase (*metE*) [22]. These enzymes add a methyl group to L-homocysteine with N^5-methyl-tetrahydrofolate (CH_3-THF) as a methyl donor.

Methionine biosynthesis in *C. glutamicum* is regulated by two ways. At the transcriptional level, it is repressed by McbR which is known to regulate the expression of diverse genes related to the oxidative stress and the biosynthesis of methionine and cysteine [23]. On the enzyme level, it is also regulated by the end-product feedback inhibition. O-acetyl-homoserine sulfhydrylase MetY is under tight feedback regulation by methionine [24].

In *E. coli*, enzymes involved in the conversion of aspartate to homoserine are aspartokinase (*lysC*, *thrA*, *metL*) [25–27], aspartate-semialdehyde dehydrogenase (*asd*) [28], and homoserine dehydrogenase (*thrA*, *metL*). Three types of aspartokinase have their own mechanisms of regulation. LysC is inhibited allosterically by lysine [29] and ThrA by threonine [30]. However, the negative feedback on MetL has not been identified yet and MetL is instead regulated by the transcriptional repressor, MetJ [31]. In addition to kinase activities, ThrA and MetL possess oxidoreductase activities functioning as homoserine dehydrogenase. Activation of homoserine is carried out by MetA [32] and results in O-succinylhomoserine (OSH). This step distinguishes *E. coli* from *C. glutamicum* in which homoserine is converted to OAH using acetyl-CoA and the enzyme, MetX. Differences from *C. glutamicum* appear in sulfur assimilation as well. Unlike *C. glutamicum*, *E. coli* has a single transsulfuration pathway using cysteine as a sulfur source and does not have direct sulfur assimilation using sulfide as a sulfur source [33]. In this step, MetB and MetC catalyze OSH to L-homocysteine using cysteine. The remaining steps are in common with *C. glutamicum*.

The role of McbR in *C. glutamicum* is performed by MetJ in *E. coli* [31]. MetJ represses the expression of a broad spectrum of genes in methionine biosynthesis including *metL*, *metA*, *metB*, *metC*, and *metH* [34]. There is a counteracting transcriptional regulator in *E. coli*, MetR [35], that upregulates *metA*, *metE*, and *metH*. Feedback inhibition by the end products, methionine and SAM, occurs in MetA [36, 37].

This section describes the metabolic pathways and regulation on methionine synthesis in two widely used microorganisms. It is often useful to compare characteristics of different strains to find ways for improvement. Therefore, we further describe metabolic pathways that are different between the two species. Section 2.2 describes the steps that activate homoserine using MetA and MetX, and Sect. 2.3 discusses enzymes assimilating sulfur. Comparisons are made particularly in the three-dimensional (3D) structures of the enzymes. To achieve industrial-scale production, it would be valuable to revisit the structure–function relationship of the key enzymes.

2.2 Enzymes for Conversion of Homoserine to O-Acylhomoserine and Their Structures

2.2.1 Homoserine Succinyltransferase

MetA (EC 2.3.1.46) carries out succinylation of homoserine using a ping–pong kinetic mechanism [38–40]. First, succinyl-CoA binds near a Cys residue in the catalytic site, which becomes more nucleophilic due to an interaction with the His residue. The Cys residue takes the succinyl group, and then CoA leaves the active site. While the succinylated intermediate is stabilized in the oxyanion hole, homoserine enters, and the succinyl group is transferred to homoserine, which completes the reaction. Activation of homoserine via succinylation is found in many enteric bacteria such as *E. coli* [8].

At present, three X-ray crystal structures of MetA are known (PDB IDs: 2GHR, 2VDJ, 2H2W). Two are of *Bacillus cereus* [40, 41] and one is of *Thermotoga maritima* (*Tm0881*) [42], which are 301 and 304 amino acids long, respectively. MetA from the two species shows about 55% sequence identity and over 70% similarity. However, the differences of the 3D backbone structures are less than 1 Å of root mean square deviation (RMSD) when excluding the 32 N-terminal amino acids. This indicates that the overall folds are highly conserved between the two species and the conformation of the active site is almost identical. However, it should be noted that MetA of *T. maritima* has dual activity as a succinyltransferase and an acetyltransferase despite the sequence annotation of MetA [43]. MetA of *B. cereus* is able to perform transacetylation exclusively by a single amino acid substitution [40]. In wild-type MetA of *T. maritima*, acetyl-CoA shows a higher turnover rate (~30-fold k_{cat}) than succinyl-CoA while having similar binding affinity. This indicates that subtle structural differences in substrate binding residues around the active site have a significant impact on the function of MetA. This will be discussed in detail at the end of this section.

Figure 2 shows MetA of *B. cereus* bound to homoserine. MetA has a Rossmann-type topology, and the catalytic triad consists of Cys142, His235, and Glu237 (nomenclature in MetA of *B. cereus*) [44–46]. Other key residues are Ala108 and Trp143 comprising the oxyanion hole and Lys163, Glu246, and Arg249 interacting with the N- and C-terminus of homoserine. The role of Lys45 and Lys46 in MetA of *E. coli* was known to be important for activity [44]. The two residues are located away from the catalytic site but adjacent to the highly flexible region (residues 75–86). MetA of *B. cereus* has disordered structure in this region, but MetA of *T. maritima* has a defined structure (Fig. 2b). This region seems to be necessary for binding of CoA or stabilizing the folds. Positions of the residues mentioned are shown in Fig. S1 of the supporting information.

MetA exists physiologically as a homodimer [41, 47]. For *B. cereus*, the 16 N-terminal residues are disordered, and structural data has not been provided by X-ray crystallography. Strikingly, residues 17–33 are well defined despite a long extended structure away from the core of the enzyme. This region together with the

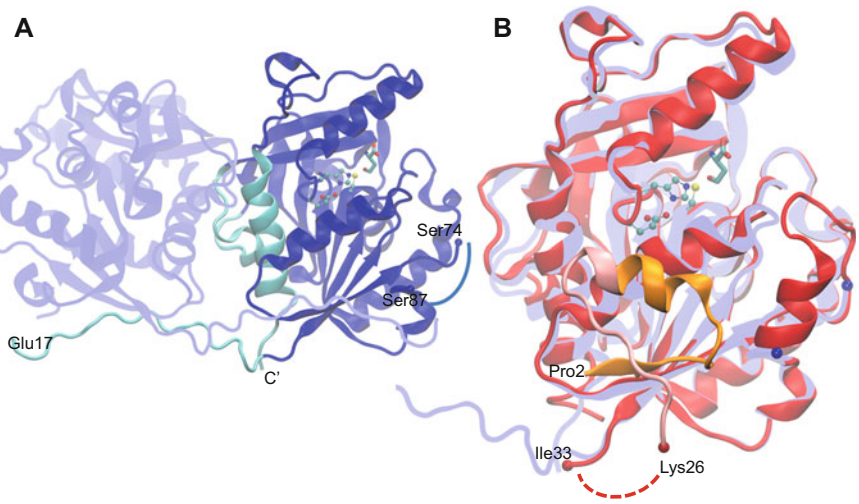

Fig. 2 Dimer of MetA and different structures of N-terminal region from MetA of *B. cereus* and MetA of *T. maritima*. MetA of *B. cereus* is shown as a *blue ribbon* and MetA of *T. maritima* as a *red ribbon*. Additionally residues 17–33, 175–186 and C-terminal helix of MetA of *B. cereus* are shown in *cyan* in (**a**), 1–16 and 17–26 of MetA of *T. maritima* in *orange* and *pink* in (**b**), respectively. Regions of missing residues in MetA of *B. cereus* are shown in *blue solid curve* and those in MetA of *T. maritima* in *red dotted curve*

unknown structure of the first 16 amino acids is important for dimerization and thermostability. Unlike MetA of *B. cereus*, MetA of *T. maritima* shows ordered structure in residues 1–16 and 17–26, and the regions interact with different residues on the surface (Fig. 2b). These differences may prevent MetA of *T. maritima* from forming an inclusion body at high temperatures [43], which has been an intriguing feature of MetA of *E. coli* [48–51]. However, the effect of such differences and the role of the N-terminal need to be further studied.

It should be noted that MetA is tightly regulated by feedback inhibition occurring at the dimerization interface which includes the N- and C-terminal regions [36, 37]. Major feedback inhibitors are D,L-methionine and SAM. A large number of point mutations have been reported to decrease the feedback inhibition. Usuda and Kurahashi [52] showed that mutations in Arg27, Ile296, and Pro298 lead to reduced feedback regulations in MetA of *E. coli*. Bestel-corre et al. [53] expanded the effective region for feedback-insensitivity to residues 24–30, 58–65, and 292–298. Independently, Leonhartsberger et al. [54] also found feedback-resistant mutations in the Asp in the AspGly$X_{aa}X_{aa}X_{aa}$ThrGlyAlaPro sequence of residues between 90 and 115 and in the Tyr in the TyrGlnX_{aa}ThrPro sequence of residues between 285 and 310. Both residues are involved in interactions near the N- and C-terminal regions. Other known mutation sites in MetA of *E. coli* [55] are Asn290 and Tyr291, which are near residues discussed above. Asn79, Glu114, Phe140, Lys163, Phe222, and Ala275 do not seem to have particular interactions with N- or

C-terminal regions but show feedback resistance [55]. Residues for mutations listed above are displayed in Fig. S2 in the supporting information.

In addition to N-terminal residues, surface residues of MetA are important regarding thermostability and acid tolerance [56]. Perturbations of electrostatics or rigidifying flexibility of surface residues are a useful strategy in enzyme engineering [57, 58]. Mutations increasing the stability of enzymes, thus leading to better cell growth, have been identified using random-mutagenesis (Ser61Thr, Glu213Val, Ile229Thr, Asn267Asp, and Asn271Lys) [56] (Fig. S3). Each residue is solvent exposed and capable of forming noncovalent interactions with nearby residues. Detailed investigation of the structure–function relationship of MetA is expected to help accelerate yields of methionine during fermentation by overcoming sensitivity to temperatures and negative feedback inhibition by end products.

There have been studies showing some MetAs are able to function as an acetyltransferase using acetyl-CoA as a substrate [8, 40, 43]. A single residue at position 111 in MetA of *B. cereus* determines the preference between succinyl-CoA and acetyl-CoA. When Gly is present at 111, the active site exclusively accommodates succinyl-CoA. However, Glu at 111 enables MetA to use acetyl-CoA and inhibits the succinyl-CoA binding by electrostatic repulsion with Glu111. This shows an interesting example in which a single point mutation can change the binding affinity of substrates and even switch the preference of substrates.

Switched substrate specificity is rare in MetX, and this phenomenon will be further discussed in Sect. 2.2.2. Structural features of MetX will be reviewed together with comparisons with MetA.

2.2.2 Homoserine Acetyltransferase

MetX (or Met2, EC 2.3.1.31) has a very similar catalytic function with MetA, but they have no close relationship in either sequence or structure. The amino acid sequence of MetX is longer than that of MetA ranging from 320 to 500 amino acids, and MetX is found in a broader spectrum of species than MetA [8].

Four structures of MetX have been identified from X-ray crystallography; MetX of *Haemophilus influenzae* (PDB ID: 2B61) [59], *Leptospira interrogans* (PDB ID: 2PL5) [60], *Staphylococcus aureus* (PDB ID: 4QLO) [61], and *Bacillus anthracis* (PDB ID: 3I1I) [62]. They differ from each other within the backbone RMSD of 3.00 Å, and major differences are in the loops on the N- and C-termini and the dimerization interface. Figure 3a highlights these regions.

MetX has a Rossmann fold in common with MetA, but its unique features include an additional five helices inserted in the middle of the Rossmann fold. This domain is called the cap or lid and was found in other enzymes containing the Rossmann fold such as aminoacrylate hydrolase [63] and 3-oxoadipate-enol-lactonase [64]. The capping domain contributes to forming not only dimers but also active sites. Figure 3b shows structures of MetX of *H. influenzae* and MetA of *B. cereus* superposed with respect to catalytic triads. Orientations of key residues

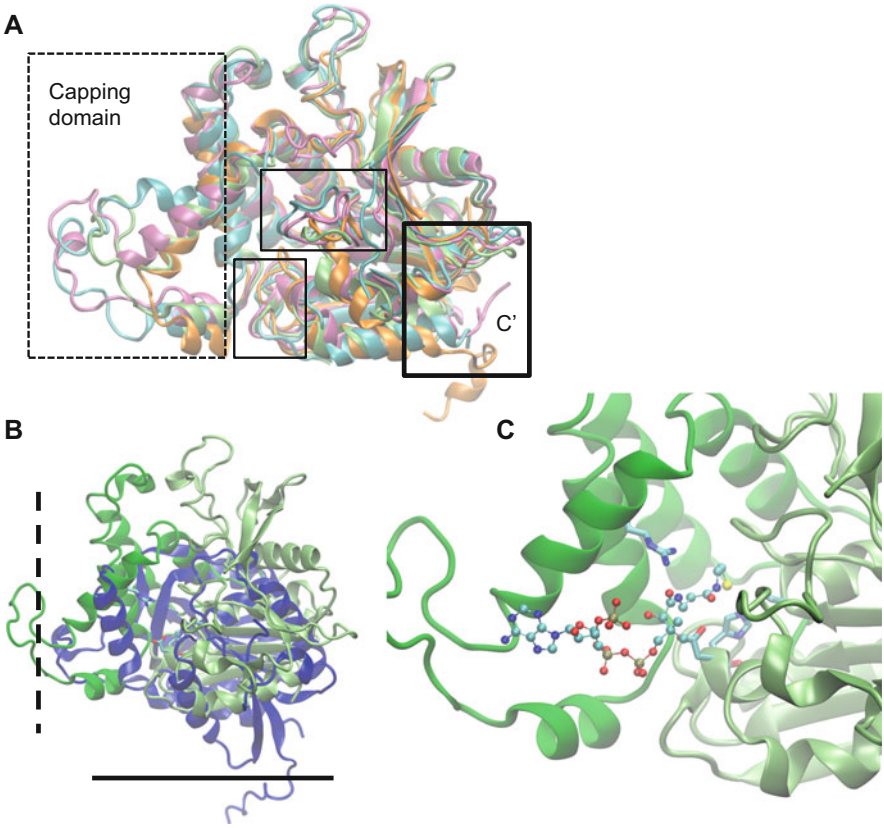

Fig. 3 Structures of MetX dimer and comparison with MetA. (**a**) MetX of *H. influenzae* is shown in *lime color* with capping domain highlighted by *dotted box*. Represented by *cyan*, *orange*, and *pink* are MetX of *L. interrogans*, MetX of *S. aureus*, and MetX of *B. anthracis*, respectively. One protomer in dimer of MetX of *H. influenzae* is not shown here. (**b**) Superposition of MetA of *B. cereus* (*blue*) on top of MetX of *H. influenzae* (*lime*) with respect to catalytic triads. Dimerization interface of MetX is posed around *dotted line* and that of MetA around *solid line*. (**c**) Putative binding site of CoA which is inferred using a structure of deacetylcephalosporin C acetyltransferase (DCA) bound to CoA. Structure of DCA and MetX are homologous and well superposed with 1.91 Å of C_α backbone RMSD

agree well with the triads (Ser143, His337, Asp304 of MetX of *H. influenzae* versus Cys148, His235, Glu237 of MetA of *B. cereus*) and two residues (Asp338, Arg212 of MetX of *H. influenzae* versus Glu246, Arg249 of MetA of *B. cereus*) interacting with the amine and carboxyl groups of homoserine, respectively. However, overall folds are still in large disagreement. MetX is different from MetA in accessibility to the active site and width of entrance. In particular, the presence of the capping domain brings different dimerization interfaces as seen in Fig. 3b. In Fig. 3c, the putative binding site of CoA is inferred from deacetylcephalosporin C acetyltransferase (PDB ID: 2VAT) [65].

Thus far, the only MetX that has been reported to have succinyltransferase activities is MetX from the *Pseudomonas aeruginosa* strain PAO1 [8, 66]. MetX of *P. aeruginosa* uses OSH instead of OAH and was complemented by MetA of *E. coli*. However, the function of MetX of *P. aeruginosa* remains to be confirmed with purified enzyme assays because its sequence is almost identical to those of MetX of *Pseudomonas putida* (82.0%) and *Pseudomonas syringae* (82.3%) which are acetyltransferases. Conversely, it will be necessary to investigate structures in detail considering the case of MetA where a difference in a single amino acid can change the function of enzymes.[1]

MetX does not show significant feedback inhibition by methionine or SAM. MetX of *Leptospira meyeri* did not affected by methionine and SAM in concentrations of 100 and 10 mM, respectively [67]. MetX of *S. cerevisiae* was inhibited by S-adenosylhomocysteine, SAM, and cysteine, but not by methionine [68]. Our in-house data (unpublished work, with Cargill) also showed that 10 mM OAH inhibits MetX of *L. meyeri* up to 35%, while methionine showed no inhibition at 12 mM in agreement with the above.

To sum up, we reviewed two enzymes, MetA and MetX, which are essential in biosynthesizing methionine precursor, O-acetylhomoserine from homoserine. The two enzymes have significantly different sequences and structures, although they share the same reaction mechanism. MetA is sensitive to temperature, easily aggregates, and is regulated by the end product, methionine. These properties of MetA have hampered the mass production of methionine. MetX has quite different characteristics from MetA. The capping domain, which consists of five helices, is unique in MetX and provides a dimerization interface. The location of the catalytic triads matches those of MetA, but the surrounding residues confer the specificity of the enzymes. MetX is relatively free from feedback regulation and structural instabilities. Hence, diversifying substrates of these two enzymes or increasing stabilities would be useful for industrial strain development.

2.3 Enzymatic Sulfur Incorporation in Methionine Biosynthesis

Sulfur assimilation is also highly variable among strains. This section focuses on recent studies exploring the structures of the sulfur-assimilating enzymes involved in methionine biosynthesis.

[1]Throughout text, sequence alignment was performed using Clustal-O and structure superposition with the FATCAT-server (rigid pairwise alignment). The VMD program was used to visualize the alignments. IDs (Uniprot) of sequences used in this review are as follows: MetA (*B. cereus*): Q72X44, MetA (*T. maritima*): Q9WZY3, MetA (*E. coli*): P07623, MetX (*P. aeruginosa*): P57714, MetX (*P. putida*): Q88CT3, MetX (*P. syringae*): Q4ZZ78, MetX (*H. influenzae*): P45131, MetX (*S. cerevisiae*): P08465, MetX (*L. interrogans*): Q8F4I0.

2.3.1 Two Sulfur Assimilation Pathways

After activation of homoserine by acylation on a hydroxyl group, the next step is to replace its acyl group with a thiol group, generating homocysteine. There are two alternative pathways. The first is the transsulfuration pathway, in which cysteine is the sulfur donor and is incorporated into the homoserine ester to form cystathionine. The reaction is catalyzed by MetB (EC 4.2.99.9), an enzyme present in most species of bacteria. This is followed by β-elimination, which cleaves cystathionine to form homocysteine, in a reaction catalyzed by MetC (EC 4.4.1.8 [69]). The net result of these two reactions is the transfer of the thiol group of cysteine to form homocysteine, through the intermediate cystathionine. Hence, this pathway is called transsulfuration. In the other pathway, the sulfur donor is free sulfide, which is incorporated into the homoserine ester in a single step by MetY (EC 2.5.1.49 O-acetylhomoserine sulfhydrylase) or MetZ (EC 2.5.1.- *O*-succinylhomoserine sulfhydrylase) to form homocysteine. This route bypasses the transsulfuration reaction and is therefore called direct sulfhydration.

All the enzymes involved are members of the Cys/Met metabolism pyridoxal-5′-phosphate (PLP)-dependent family, which is a subclass of the PLP-dependent fold-type I proteins [70]. Therefore, these enzymes show sequence similarities, and their 3D structural folds are highly similar.

Kim et al. [71] evaluated activities of MetB, MetY, and MetZ. The degree of difference in these enzymes is not as dramatic as between MetA and MetX. Various MetY and MetZs are able to utilize both OSH and OAH as their substrate. In addition, many of these enzymes use cysteine, methanethiol, and sulfide as the sulfur source, with different degrees of efficiency. Notably, despite the annotation as MetZ, MetZ of *H. neptunium* showed higher specificity for OAH than for OSH. These results also corroborate the previous findings that various organisms evolved to produce just one of the homoserine-esterified substrates but that their enzymes retain the ability to use other homoserine-esterified substrates as well [72]. Nonetheless, it is important to point out structural elements that confer preferences in substrates. Section 2.3.2 overviews common characteristics in MetB, MetY, and MetZ, and Sect. 2.3.3 discusses structural analysis of the differences.

2.3.2 Cystathionine-γ-Synthase and O-Acylhomoserine Sulfhydrylases: Members of the Same Superfamily

MetB, MetY, and MetZ are homologous and all require PLP for activity. These enzymes are tetrameric proteins of the identical subunit with one PLP cofactor bound per monomer via a Schiff base linkage to an active site lysine (Fig. 4a). Thus, each monomer has an active site that requires an N-terminal domain of another monomer via salt bridges to the phosphate group of PLP. In the resting state, PLP is covalently bound to the amino group of an active site lysine, forming an internal aldimine. Once the substrate O-acetylhomoserine interacts with the active site, a

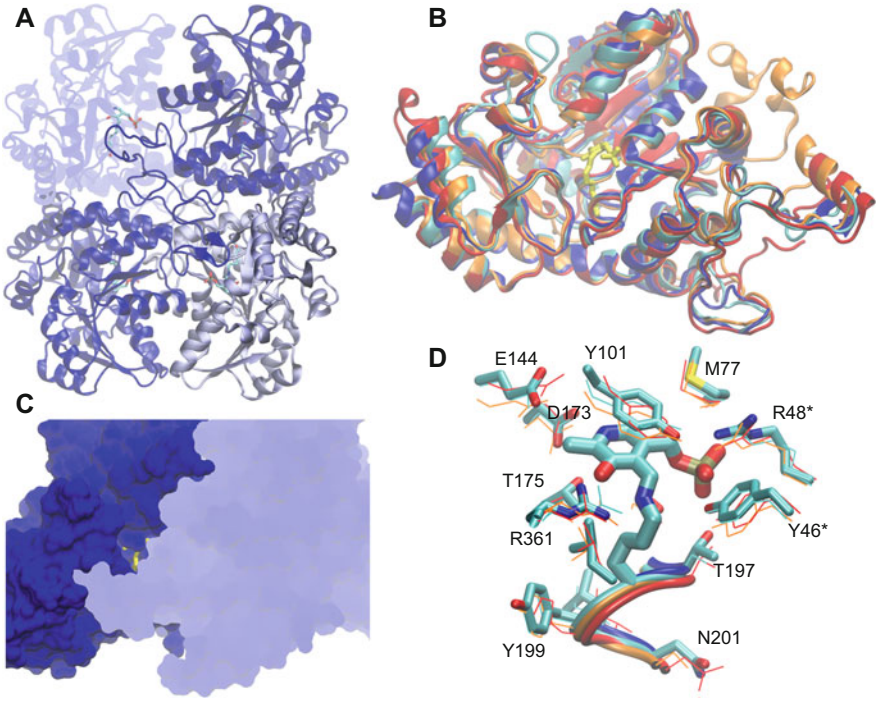

Fig. 4 Structures of MetB, MetY and MetZ. (**a**) Tetramers with PLPs bound to each monomer (MetB of *E. coli*). PLP is represented as stick model. (**b**) Superposition of monomers from four different enzymes; MetB of *E. coli* is shown in *blue*, MetB of *M. ulcerans* in *cyan*, MetZ of *M. tuberculosis* in *red* and MetY of *C. jejuni* in *orange*. PLP in the active site is shown in *yellow*. (**c**) Substrate access channel formed in dimerization interface (MetB of *E. coli*). (**d**) Highly conserved active site residues in four enzymes. Residues indicated are from MetB of *E. coli* and shown in the thick stick model while corresponding residues from other three enzymes are shown in the thin stick model with the color scheme used in (**b**). * Indicates residues from the neighboring monomer

new Schiff base is generated commonly referred to as an external aldimine, thereby allowing the acetyl or succinyl group to be eliminated. In this step, PLP acts as an electron sink to stabilize carbanion intermediates. This results in a PLP-bound intermediate with a vinyl side chain, which is then attacked by the second substrate – cysteine, hydrogen sulfide, or methanethiol – to yield cystathionine, homocysteine, or methionine, respectively. Each monomer is composed of three subdomains. An N-terminal domain has an extended loop structure, which protrudes from the bulk of the monomer, forming a clamp to the neighboring monomer. The large PLP-binding domain with a mostly parallel seven-stranded β-sheet at its center contains the active site. The C-terminal domain with a slightly twisted antiparallel β-sheet is packed against the PLP-binding domain, contributing to the formation of the compact monomer shape.

Figure 4b showed superposed MetB of *E. coli* with three other structures to check the overall similarities. MetB of *E. coli* (PDB ID: 1CS1 [73]) was aligned to MetB of *Mycobacterium ulcerans* (PDB ID: 3QI6, 3QHX [74]), MetY of *Campylobacter jejuni* (PDB ID:4OC9 [75]), and MetZ of *Mycobacterium tuberculosis* (PDB ID: 3NDN [76]) with less than 1.8 Å of C_α backbone RMSD. All secondary structural elements are well superposed with a few exceptions (Fig. 4b).

The active site appears as a relatively large cavity open to solvent in the monomeric form, but an access channel for substrates is formed in the dimer (Fig. 4c). PLP is covalently attached via a Schiff base linkage to Lys198 (nomenclature of MetB of *E. coli*). Besides the covalent bond, the cofactor is anchored predominantly in the active site through its phosphate group, through which several hydrogen bonds interact with protein residues, including the side chains of Tyr46*, Arg48* by charge–charge interactions. The equivalent residues to Tyr101 of MetB form π-stacking interactions with the pyridine ring of PLP. Together with Tyr101, Thr175 and Ser195 sandwich PLP in the active site. The catalytically important Asp173 forms a salt bridge to the PLP pyridine nitrogen (N1), thereby increasing the electrophilic propensity of PLP. Besides Lys198 and Asp173, the conserved Arg361 is assumed to interact with the α-carboxylate group of the incoming substrate. The positions of key conserved residues mentioned above (Lys198, Tyr101, Tyr46*, Arg48* and Asp173) are closely aligned (Fig. 4d). An inhibitor and SO_4^{2-} molecules bound to MetB of *M. ulcerans* suggest possible positions of acyl groups in the substrates [74] (see Sect. 2.3.3).

2.3.3 Structure–Function Relationships

Despite having highly conserved active sites, there are regions of divergence within the overall structures of the various enzymes. One of major difference is shown in the substrate access channel. In MetB of *E. coli*, the regions consist of residues 339–358 from one monomer and residues 30–50 and 215–230 from an adjacent monomer (Fig. 5a). A larger cavity is formed in MetB of *E. coli* than in MetY and MetZ. This is due to the three regions being arranged farther from each other, and residues 30–50 include coils in MetBs, while MetY and MetZ have an additional helix protruding toward the active site. Usually MetY and MetZ have longer sequences in this region based on sequence alignment (Fig. S5). MetY of *C. jejuni* has longer structures near residues 215–230 of MetB of *E. coli*, and it causes an even narrower channel to the active site. Interestingly, in MetZ positive charges are found near the entrance to the active site (Fig. 5b). In MetZ, either Arg or His is positioned toward the SO_4^{2-} ion, which is considered to be the position of the succinate group of OSH. It is the same in MetB of *E. coli* and *M. ulcerans*, which use OSH as a substrate. MetY has an Asp or Gln on the corresponding position.

The differences observed above may determine preferences for substrates with different sizes and electrostatics. However, comparative data on substrate specificity in these four enzymes is required to establish detailed structure–function

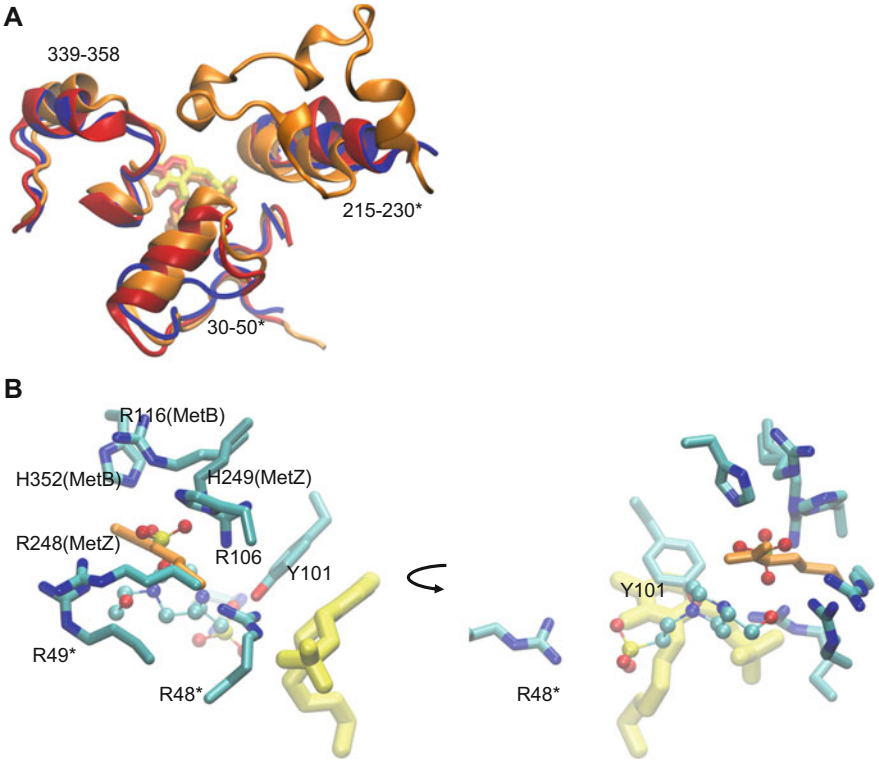

Fig. 5 (**a**) Three regions showing variations of sequences and structures in MetB of *E. coli*, MetZ of *M. tuberculosis* and MetY of *C. jejuni*. The same superposition and color scheme were used as Fig. 4b. *Left region* covers residues 339–358 in monomer A. In *upper right region* are residues 215–230 in monomer B, and helix in *orange* (MetY of *C. jejuni*) is particularly tilted toward active site. Region on the *center of bottom* indicates residues 30–50 in monomer B and it contrasts that helical structures of MetY and MetZ and coiled structures of MetB. (**b**) Positively charged residues around SO_4^{2-} molecule which is presumed to be a binding site of succinyl moiety. While MetB and MetZ utilizing OSH have His or Arg residues, MetY utilizing OAH does not show such residues. With this lack of interactions it is possible that OAH binding more favorably. Arg represented in *orange stick* is from MetY but it lies across the active site and with conformational changes it can superpose with R248 (MetZ) and R49 (MetB of *E. coli*). SO_4^{2-} ion and an inhibitor 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid are shown in the ball-and-stick model. Residues from MetB of *M. ulcerans* are indicated as MetB in *parenthesis* and MetZ of *M. tuberculosis* as MetZ. Otherwise residues are from MetB of *E. coli*

relationships. In summary, sulfur-assimilating enzymes have high structural similarities but show different substrate preferences with flexibility on other substrates. Engineered enzymes would make it possible to utilize diverse sulfur sources and each O-acylhomoserine more efficiently. Examining structural elements responsible for feedback inhibition by methionine in MetY could be another target. Structural investigations comparing MetY with MetB and MetZ could be one solution because MetZ has no or highly limited feedback regulation by methionine [71].

Next, we shift our attention from individual enzymes to whole cells. Functions and regulations of purified enzymes may behave in unexpected ways in specific microbial strains where hundreds of metabolic pathways are signaling to one another. Therefore, it is recommended that characterization of enzymes and strain development are performed simultaneously.

3 Strain Development

For industrial production of methionine through fermentation, the process must be as economical as chemical methods. Therefore, development of appropriate strains is of the utmost importance. Diverse microorganisms have been studied and one of the most common techniques is classical mutagenesis [77]. Microorganisms are modified using mutagens such as methylnitronitrosoguanidine and ultraviolet radiation and then selected by the resistance to a high concentration of amino acid analogues. Currently, strains are primarily developed through genetic modifications based on metabolic engineering [13], which is more specific and targeted than classical mutagenesis. This section reviews methods used to develop microorganisms producing methionine and how such efforts improve the yield of methionine fermentation.

3.1 Strain Development for Methionine Production

3.1.1 Classical Mutagenesis

Traditionally, mutagenesis has been used for screening mutants that are resistant to amino acid analogues. To find methionine-producing strains, classical mutagenesis was applied using ethionine, selenomethionine, norleucine, and methionine hydroxamate as analogues [78]. Mutants of *Brevibacterium heali* were screened based on ethionine resistance and were reported to yield 4–25.5 g/L of methionine [79]. Resistant mutants to multi-methionine analogues were also screened in *Corynebacterium lilium* [80] and *Bacillus megateria* [81] and synthesized 2–4.5 g/L of methionine. A strain of *E. coli*, generated among mutants resistant to threonine and methionine analogues, was reported to produce 2 g/L of methionine [82, 83].

However, one study [84] called into question possible errors in the reported concentrations of methionine. The study asserts that the amount of methionine produced was higher than the theoretical yields that could be obtained from the sulfur contained in the production media. Moreover, the amount of methionine, even if it was reported correctly, is still far lower than required for mass production. Classical mutagenesis methods paved the way for the methionine fermentation but these techniques still have too many limitations.

3.1.2 Metabolic Engineering

Strain development has recently evolved using rational design employing metabolic engineering. *E. coli* and *C. glutamicum* are the best candidates for methionine production since they have been successfully used for the industrial fermentation of other amino acids.

Theoretical yields of methionine are 52 and 49.3% in *E. coli* and *C. glutamicum*, respectively, using sulfate as a sulfur source [85]. Differences in the theoretical yields are closely related to the NADPH production in the two species. When reducing sulfate to sulfide, two ATP and four NADPH are consumed. Therefore, methionine needs four additional NADPH molecules compared with lysine which does not incorporate sulfur. In total, eight NADPH molecules are used to produce methionine. *E. coli* generates NADPH by transhydrogenase [86], while *C. glutamicum* depends on pentose phosphate pathways [87]. In the pentose phosphate pathway, CO_2 loss is inevitable and leads to lower theoretical methionine yields. However, the gap between the two species could be overcome by introducing key genes and should not be a problem during the strain development.

The theoretical yield of methionine is most affected by the sulfur source since sulfur assimilation needs high energy and high reducing power [85, 88] (Fig. 6). Sulfate and thiosulfate are oxidized sulfurs, which can be directly used for fermentations, and the methionine yields could be 49.3 and 60.8%, respectively, in *C. glutamicum*. As described in Fig. 6, thiosulfate saves more energy and reducing power than sulfate, which increases the yield. The yields could be further improved with reduced sulfur such as hydrogen sulfide (H_2S) and methanethiol up to 67.8 and 90.0%, respectively. However, H_2S and methanethiol are highly volatile, flammable, and toxic, which properties are not applicable to fermentation using large amounts of air. Therefore, thiosulfate is regarded as the most optimal sulfur source in the methionine fermentation. Table 1 summarizes calculated methionine yields in *E. coli* and *C. glutamicum* with different sulfur sources.

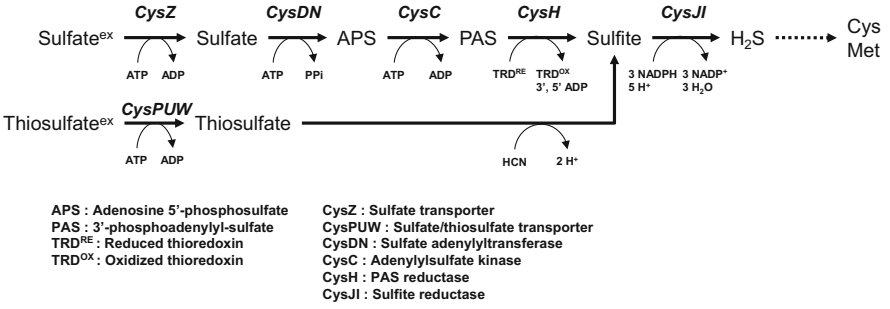

Fig. 6 Sulfur assimilation pathway

Table 1 Theoretical yields of methionine with different sulfur sources [85]

Strain	Genetic modification	Sulfur sources	Additional C1 source	Theoretical yield [%C]
E. coli	–	Sulfate	–	52.0%
	Lacking transhydrogenase	Sulfate	–	49.9%
	Lacking glycine cleavage system	Sulfate	–	45.5%
C. glutamicum	–	Sulfate	–	49.3%
	Active transhydrogenase	Sulfate	–	49.5%
	Active glycine cleavage system	Sulfate	–	57.1%
	–	Thiosulfate	–	60.8%
	–	Thiosulfate	Formate	66.3%
	–	Sulfide	–	67.8%
	–	Sulfide	Formate	74.8%
	–	Sulfate	Formate	52.1%
	–	Methanethiol	–	90.9%

3.2 *Methionine Production Yield in Previous Studies*

3.2.1 *E. coli* Engineering

E. coli is certainly one of the most useful microorganisms because of the abundance of genetic information and engineering tools. In this section, we list four examples of strain developments performed with *E. coli*.

Nakamori et al. [89] achieved a yield of 0.236 g/L of methionine using the following approach. In the *E. coli* W3110 strain, MetJ, methionine repressor and ThrB, ThrC, which competes for homoserine, were removed. Furthermore, the activity of MetK was reduced to prevent methionine degradation. Next, MetA was overexpressed with mutations in Arg27Cys, Ile296Ser, and Pro298Leu to suppress the feedback inhibition.

Another approach yielded 4.8 g/L of methionine by enhancing methionine efflux through overexpression of the YjeH transporter in the *E. coli* W3110 strain, with a feedback-resistant MetA (Tyr294Cys) expression and *metJ* deletion [90].

When starting from the threonine-producing mutant strain, *E. coli* TF4076, 6.6 g/L of methionine production was reported [55]. Similar to the prior two examples, the *metJ* and the *thrB* genes were deleted, and feedback-resistant MetA (Asn290His) was overexpressed. Additionally, MetF, which acts in the methyl-THF pathway, and MetX and MetY, derived from *L. meyeri*, were overexpressed.

E. coli MG1655 was developed as well to produce 35 g/L of methionine corresponding to a 20–24% of yield ($g^{Met}/g^{Glucose}$) [53]. The *metJ* gene was deleted and *metA* was overexpressed with mutations abolishing feedback inhibition. *thrA*

was also overexpressed with reduced feedback inhibition by threonine. Other genes with increased activities were *metF*, *metH*, *gcVTHP*, and *glyA* in the C1 metabolism pathways; *cysE*, *serA*, *serB*, and *serC* in the cysteine biosynthesis pathways; and *cysPUWAM* and *cysJIH* in the sulfur assimilation pathways.

3.2.2 *C. glutamicum* Engineering

C. glutamicum is well known as a strain for glutamate and lysine fermentation and several studies are ongoing for methionine production.

C. glutamicum DSM 5715 derived from an S-2-aminoethyl-cysteine-resistant lysine producer was reported to produce 16 g/L of methionine by the overexpression of *metX* and *metY* [91]. Another lysine producer, *C. glutamicum* MH20-22B (leu^-, $lysC^{FBR}$) was modified to produce 2.9 g/L methionine by deleting *thrB* and introducing hom^{FBR} [92].

Zelder et al. [93] used *C. glutamicum* ATCC13032 for enhanced methionine production as well. *lysC* and *hom* with feedback resistance were introduced and homoserine kinase (*hsk*) was weakened to suppress the competing pathway. Overexpressed genes were *metH*, *metY*, and *metX*, which are related to methionine biosynthesis, and *metF* in the methyl donor pathway. Deletions were made in a transcriptional repressor (*mcbR*), a methionine uptaker (*metQ*), and the vitamin B_{12} uptake repressor (*btuR2*). As a result, the strain yielded 6.8 g/L of methionine.

Slightly different approaches were taken with *C. glutamicum* ATCC13032 to achieve 6.3 g/L of methionine by fed-batch fermentation. In this strain, *thrB* and *mcbR* were deleted, feedback-resistant *lysC* was introduced, and *BrnFE* was overexpressed to efflux methionine [94].

In summary, the strain showing the best performance was *E. coli* MG1655 by Bestel-corre et al. [53]. However, the production efficiency by this method is still far behind the commercial production scale.

3.3 Semifusion or Fusion Process for Methionine Production

Major huddles in methionine biosynthesis are high energy demands, high requirement for the reducing power, and complexity of the methyl donor pathways (C1 metabolism). Furthermore, intermediates such as homocysteine are toxic to the host cells thereby causing cells to develop regulating mechanisms for self-protection [95]. Thus, strain development still has a long way to go to catch up to the chemical synthesis from an economic standpoint. However, there have been several studies to narrow this gap using semifusion or fusion fermentation processes.

The first attempt was the use of reduced sulfur in the fermentation to overcome issues of ATP and NADPH [85, 88, 96–99]. The theoretical methionine yield is 90.9% with methanethiol, by the donation of methyl group and sulfur together. As

an analogue of methanethiol, dimethyl disulfide (DMDS) was studied in *C. glutamicum* [99]. After deleting *metB*, *metF*, *metE* (or *metH*), *C. glutamicum* was still viable without external methionine when given DMDS. It was determined that DMDS is able to act as a substrate of MetY and that DMDS could be another sulfur source for methionine. DMDS was released to *C. glutamicum* slowly using oils as a delivery system due to a high rate of evaporation. By this method, the M2014 strain (*hom*^{FBR}, *lys*^{FBR}, *ethionine*^{R}, *thrB*^{reduced}, *metH*, *metY*, *metX* overexpression) produced 0.5–0.7 g/L of methionine. However, toxicity is still unavoidable with reduced sulfur. This method showed the growth inhibition at 0.8% DMDS and It would be important to overcome the toxicity of reduced sulfur in the future.

Another approach is a combination of fermentation and enzymatic conversion. After synthesizing the methionine precursor, O-acylhomoserine by fermentation, reduced sulfur can be assimilated through enzymatic conversion to produce methionine [55]. O-acylhomoserine synthesis does not use sulfur assimilation pathways or C1 metabolism. Thus, it costs less energy and reducing power and it can avoid tight cellular regulation by methionine. Furthermore, enzymatic conversion is free from sulfur toxicity because it is performed without living cells. Theoretical yield of OAH is 80.3% ($g^{OAH}/g^{Glucose}$), and the enzymatic conversion yield is 92.5% (g^{Met}/g^{OAH}) by MetY with OAH and methanethiol as substrates. As such, the overall theoretical yield of methionine becomes 74.3% ($g^{MET}/g^{Glucose}$). This value is lower than using methanethiol directly during the fermentation but it is a promising approach economically since byproducts acetate can be recycled as a carbon source. When using OSH as a precursor, the yield is 81.2% ($g^{OSH}/g^{Glucose}$) for OSH and 68.0% (g^{MET}/g^{OSH}) for enzymatic conversion and overall 55.2% ($g^{Met}/g^{Glucose}$) for methionine. In terms of methionine yield, it is less productive than OAH but it is an attractive pathway in that 1 mol of methionine produces 0.8 mol of succinate as a byproduct, which has potential as a bio-based C4 chemical. It is expected that the two methionine precursors – OSH and OAH – could be used interchangeably according to economic trends (Fig. 7).

This two-step process was industrialized by CJ CheilJedang-Arkema with 80,000 tons per year production capacity in 2014 [6], which is the first L-methionine industrial production by fermentation. Here is a brief description of the two-step process based on Kim et al. [71]. *E. coli* strain producing OAH was prepared by the deletion of *metB*, *thrB*, and *metA* to maximize the accumulation of homoserine with the overexpression of *metX*. The strain yielded OAH greater than 55 g/L in a fed-batch fermentor. In this step, MetA of *E. coli* with Gly111Glu point mutation could be used instead. As previously mentioned, this mutation can change the product from OSH to OAH, which can make a *E. coli* host strain without insertion of foreign genes [100].

In second step, enzymes converting OAH to methionine were selected in two criteria: substrate specificity and feedback resistance by methionine. Various enzymes from microorganisms were analyzed in terms of OAH or OSH, using methanethiol as a sulfur source. It was found that several enzymes were able to utilize both OSH and OAH as substrates with different degrees of efficiencies.

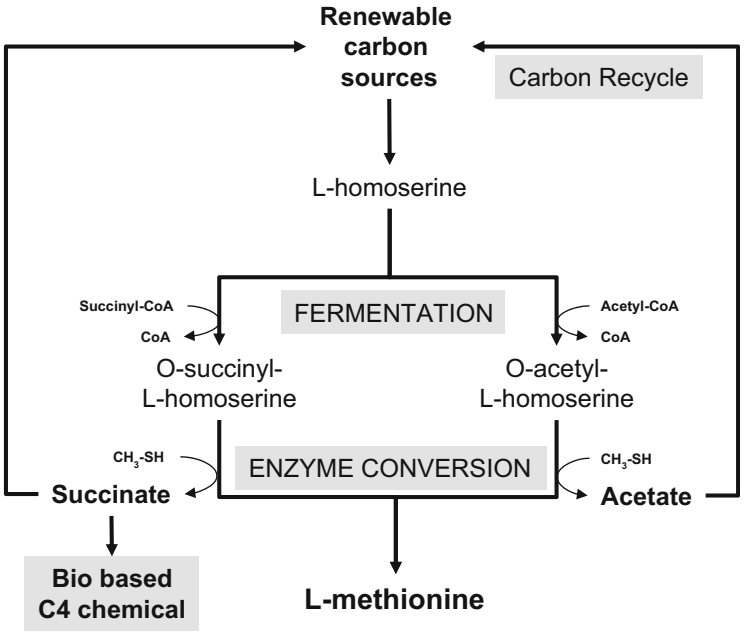

Fig. 7 Semi or fusion process

Notably, some enzymes annotated as MetZ have higher specificity for OAH than for OSH. Then, feedback inhibition of these enzymes was analyzed in vitro. It was found that the product inhibition of MetZs is relatively less than that of MetYs. Residual activities of MetYs from *P. aeruginosa* and *L. meyeri* were approximately 50%, while MetZs from *P. putida*, *C. violaceum*, and *H. neptunium* showed over 90% of residual activities in the presence of 5 g/L methionine. Considering these aspects, MetZ was the best candidate because it has less product inhibition and high acceptability of methanethiol and OAH. Methionine could be produced with MetZ derived from *Hypomonas* sp. with a yield of 40 g/L using OAH fermentation broth and methanethiol, which was the highest concentration reported [71].

4 Conclusion

Since the 1950s, processes for synthesizing DL-methionine chemically have been optimized and have become the most competitive commercial process. During the last 30 years, research efforts toward improving L-methionine fermentation have been made, but there still remains some gap between our understanding of microorganisms and amino acid mass production. Here, we briefly review the enzymes related to the O-acylhomoserine production and sulfur assimilation. An interesting point is that homoserine succinyltransferase (MetA) has tight feedback inhibition,

while OSH sulfhydrylase (MetZ) has little feedback inhibition with broad substrate specificity. However, homoserine acetyltransferase (MetX) has no feedback inhibition while OAH sulfhydrylase (MetY) has strong feedback inhibition with tight substrate specificity. Hence, at least one enzyme of each pathway – OAH or OSH – retains tight regulation, which is one of the major hurdles in the methionine production. However, these characteristics could be a good starting point for avoiding methionine regulation by the combination of two enzymes of each pathway. Two-step process using MetX and MetZ in OAH pathway could be one example for this combination, and there might be more opportunities for this kind of mixture.

There are still many aspects of these enzymes that have not yet been discovered. With a sustained study on the structure–function relationships of these enzymes accompanied by a metabolic engineering for host strains, it is anticipated that L-methionine fermentation technologies will grow to be competitive with chemical synthesis in the foreseeable future.

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Part III
Recent Advances in Amino Acid Fermentation Research

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Boosting Anaplerotic Reactions by Pyruvate Kinase Gene Deletion and Phosphoenolpyruvate Carboxylase Desensitization for Glutamic Acid and Lysine Production in *Corynebacterium glutamicum*

Atsushi Yokota, Kazunori Sawada, and Masaru Wada

Abstract In the 1980s, Shiio and coworkers demonstrated using random mutagenesis that the following three phenotypes were effective for boosting lysine production by *Corynebacterium glutamicum*: (1) low-activity-level citrate synthase (CS^L), (2) phosphoenolpyruvate carboxylase (PEPC) resistant to feedback inhibition by aspartic acid ($PEPC^R$), and (3) pyruvate kinase (PYK) deficiency. Here, we reevaluated these phenotypes and their interrelationship in lysine production using recombinant DNA techniques.

The *pyk* deletion and $PEPC^R$ (D299N in *ppc*) independently showed marginal effects on lysine production, but both phenotypes synergistically increased lysine yield, demonstrating the importance of PEPC as an anaplerotic enzyme in lysine production. Similar effects were also found for glutamic acid production. CS^L (S252C in *gltA*) further increased lysine yield. Thus, using molecular techniques, the combination of these three phenotypes was reconfirmed to be effective for lysine production. However, a simple CS^L mutant showed instabilities in growth and lysine yield.

Surprisingly, the *pyk* deletion was found to increase biomass production in wild-type *C. glutamicum* ATCC13032 under biotin-sufficient conditions. The mutant showed a 37% increase in growth (based on OD_{660}) compared with the ATCC13032 strain in a complex medium containing 100 g/L glucose. Metabolome analysis revealed the intracellular accumulation of excess precursor metabolites. Thus, their conversion into biomass was considered to relieve the metabolic

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distortion in the *pyk*-deleted mutant. Detailed physiological studies of various *pyk*-deleted mutants also suggested that malate:quinone oxidoreductase (MQO) is important to control both the intracellular oxaloacetic acid (OAA) level and respiration rate. These findings may facilitate the rational use of *C. glutamicum* in fermentation industries.

Keywords Anaplerotic pathway, Citrate synthase, *Corynebacterium glutamicum*, Feedback inhibition, Glutamic acid, Lysine, Malate:quinone oxidoreductase, Phosphoenolpyruvate carboxylase, Pyruvate kinase, Respiration

Contents

1 Contribution of Anaplerotic Reactions to Lysine Yield: Phosphoenolpyruvate Carboxylase vs. Pyruvate Carboxylase

1.1 Introduction

The anaplerotic reaction that replenishes oxaloacetic acid (OAA) is important for fermentative production of lysine because OAA, a precursor metabolite for lysine biosynthesis, is continuously withdrawn from the tricarboxylic acid (TCA) cycle during lysine production. In other words, an efficient anaplerotic reaction is required to increase lysine yield. Two anaplerotic reactions operate in *Corynebacterium glutamicum*: the formation of OAA from phosphoenolpyruvate (PEP) by phosphoenolpyruvate carboxylase (PEPC) and that from pyruvic acid by pyruvate carboxylase (PC). The contributions of these two reactions to lysine production have been investigated in detail for over 3 decades. Here, a brief history of these studies is provided.

1.2 Phosphoenolpyruvate Carboxylase Reaction for Lysine Production

In the 1980s, Shiio and coworkers were the first to shed light on the PEPC reaction for lysine production [1]. Using repeated random mutagenesis induced by *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (NTG) treatment, they demonstrated enhanced lysine yields in *Brevibacterium flavum* (later reclassified as *C. glutamicum*) mutants having three distinct phenotypes: (1) low-activity-level citrate synthase (CS^L), (2) PEPC that is resistant to feedback inhibition by aspartic acid ($PEPC^R$), and (3) a pyruvate kinase (PYK) defect, in either a feedback-inhibition-resistant aspartokinase (AK^R) background or a null-activity homoserine dehydrogenase background, suggesting the contribution of PEPC to catalyzing an anaplerotic reaction in lysine production. A PYK-defective mutant, KL-18, derived from an AK^R-type lysine producer, No. 2-190, exhibiting both CS^L and $PEPC^R$, is a representative example of the importance of PEPC as an anaplerotic enzyme for lysine production ([2], Table 1). As shown in Fig. 1, $PEPC^R$ may contribute to improved lysine production, presumably through a smooth supply of OAA from PEP. A mutation causing PYK deficiency may block the metabolism of PEP to pyruvic acid, thereby directing the conversion of PEP into OAA by $PEPC^R$. On the other hand, CS^L is expected to decrease the consumption of OAA, enabling more OAA and pyruvic acid to be used for lysine biosynthesis. However, as these studies were conducted using mutants derived randomly by repeated NTG treatment, the precise contribution of each mutation to lysine production and the interrelationship among these mutations remain to be elucidated.

Table 1 Effects of PYK mutation on lysine production in various *C. glutamicum* strains

Strain	Relevant mutation	Lys productivity	Reference
Brevibacterium flavum			
No. 2-190	CS^L, $PEPC^R$, AK^R	38 g/L (as Lys·HCl)	Shiio et al. [2]
KL-18	PK-defective mutant of No. 2-190[a]	43 g/L (as Lys·HCl)	
Corynebacterium lactofermentum			
ATCC21799	Lys producer, AEC^R [b]	26.3 g/L	Gubler et al. [7]
L124	*pyk*-gene disruptant of ATCC21799	15.0 g/L	
Corynebacterium glutamicum			
ATCC21253	Lys producer, *hom*, *leu*[c]	20 g/L	Park et al. [8]
SM575	*pyk*-gene disruptant of ATCC21253	14 g/L	
Corynebacterium glutamicum			
ATCC13032 $lysC^{fbr}$	$lysC^{fbr}$ [d]	10 mM (from 80 mM Glc.)	Becker et al. [9]
ATCC13032 $lysC^{fbr}$ ΔPYK	*pyk*-gene deletion of ATCC13032 $lysC^{fbr}$	6 mM	

[a]Derived by *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine treatment
[b]AEC^R, S-(2-aminoethyl)-L-cysteine resistance
[c]*hom*, *leu*, auxotrophic for homoserine and leucine, respectively
[d]$lysC^{fbr}$, feedback-desensitized aspartokinase gene with T311I mutation

1.3 Pyruvate Carboxylase as an Important Anaplerotic Enzyme for Lysine Production

From the early 1990s, the development of genetic engineering enabled more precise metabolic analysis in *C. glutamicum*. Moreover in 1997, the activity of PC, another anaplerotic enzyme, was detected for the first time in this species using a permeabilized cell preparation [3]. This promoted identification of the anaplerotic reaction that contributes to lysine production in this bacterium. Specifically, for lysine production, PC but not PEPC was found to be important as a major anaplerotic enzyme supplying OAA; knockout of the PEPC gene did not affect lysine production [4], overexpression of the PC gene enhanced it, while inactivation of the PC gene led to a decrease in lysine yield [5]. Moreover, the positive effects of PYK-defective mutation on lysine production as reported by Shiio and coworkers [2, 6] were not observed, and even negative effects were detected in three independent studies in which *pyk*-gene knockout mutants were generated by genetic engineering [7–9]. As listed in Table 1, introduction of a *pyk* knockout mutation to various lysine producers having either AK^R or homoserine auxotrophy led to decreased lysine productivity, to approximately 50% of those of the corresponding

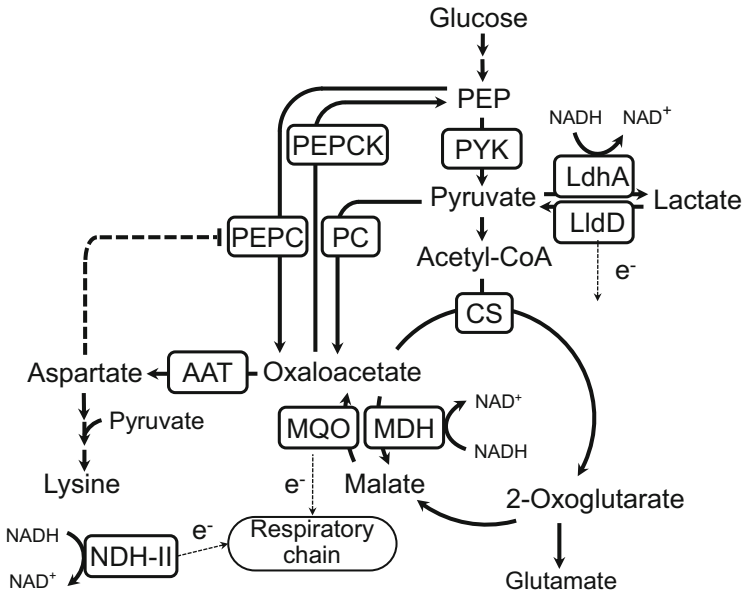

Fig. 1 Metabolic pathways focusing on anaplerotic reactions and respiration-related reactions in *Corynebacterium glutamicum*. *PEP* phosphoenolpyruvate, *PYK* pyruvate kinase, *PEPC* phosphoenolpyruvate carboxylase, *PEPCK* phosphoenolpyruvate carboxykinase, *PC* pyruvate carboxylase, *AAT* aspartate aminotransferase, *CS* citrate synthase, *LdhA* lactate dehydrogenase, *LldD* quinone-dependent lactate dehydrogenase, *MQO* malate:quinone oxidoreductase, *MDH* malate dehydrogenase, *NDH-II, NADH* dehydrogenase-II, e^- electron. The *dashed line* denotes feedback inhibition

parents. Under these conditions, PC has generally been recognized as an important anaplerotic enzyme for lysine production; the P458S mutation in *pyc* (enhanced PC activity) [10] has often been employed to construct lysine producers. Until recently, these contradictions concerning the effect of PYK mutation on lysine production were ignored. However, these discrepancies strongly suggested the importance of the coexistence of PYK defect and $PEPC^R$ for the enhancement of lysine production, as reported by Shiio and coworkers in their work using random mutagenesis [2, 6].

1.4 Refocusing on Phosphoenolpyruvate Carboxylase for Lysine Production

Although the PEPC reaction had been overlooked regarding lysine production, recently, PEPC was again demonstrated to be important for lysine production by a study in which its feedback inhibition by aspartic acid was deregulated [11]. Incidentally, a recent molecular technique study also demonstrated the effectiveness of

decreased CS activities on lysine production in *C. glutamicum* [12]. Therefore, the interrelationships among mutations conferring the aforementioned phenotypes described by Shiio and coworkers, namely, CS^L, $PEPC^R$, and PYK defect, on lysine production need to be reevaluated using modern recombinant DNA techniques.

2 Effects of Pyruvate Kinase Mutation on Glutamic Acid and Lysine Production in the Presence or Absence of Phosphoenolpyruvate Carboxylase-Desensitizing Mutation

2.1 *Introduction*

As described in the previous section, *pyk* knockout or deletion [7–9] and $PEPC^R$ [11] generated by recombinant DNA techniques were independently identified to have negative and positive effects on lysine production, respectively. However, the combined effect of these mutations on lysine production had not been clarified. In addition, no reports have described the effect of either *pyk* deletion or $PEPC^R$ on glutamic acid production. Thus, we have investigated the effects of *pyk* deletion on glutamic acid and lysine production in the presence or absence of the $PEPC^R$ mutation. In addition, the impact of CS^L on lysine production, which Shiio and coworkers [13] suggested to have a promoting effect, was also evaluated using recombinant DNA techniques.

2.2 *Effects of* pyk *Deletion and Phosphoenolpyruvate Carboxylase Desensitization on Glutamic Acid Production*

We derived a *pyk*-deleted mutant (strain D1) from wild-type *C. glutamicum* ATCC13032 using the double-crossover chromosome replacement technique and investigated its glutamic acid productivity under biotin-limited conditions [14]. When cultured in a 2-L jar fermentor using Medium F4 (complex medium with 100 g/L glucose and 3 μg/L biotin), strain D1 produced 25% more glutamic acid (32.0 g/L) than did ATCC 13032 (25.6 g/L) (Table 2). This was the first demonstration of the primary effects of *pyk* deletion in a defined *C. glutamicum* mutant.

An amino acid residue involved in $PEPC^R$ in *C. glutamicum* ATCC13032 was next identified based on the PEPC sequence information of *C. glutamicum* No. 70 [15]. A single amino acid substitution in PEPC, D299N, was found to relieve the feedback inhibition by aspartic acid. A simple mutant, strain R1, having this D299N substitution in PEPC was constructed from ATCC13032 using the same double-crossover chromosome replacement technique as in the case of strain D1.

Table 2 Effects of *pyk* deletion on glutamic acid and lysine production in *C. glutamicum*

Strain	Relevant mutation	Glutamic acid (g/L)	Aspartic acid (g/L)	Lysine (g/L)	Reference
Glutamic acid producer					
ATCC13032	Wild-type	25.6	0.44	–[a]	Sawada et al. [14]
D1	Δ*pyk*	32.0	2.3	–	
R1	$PEPC^R$	31.0	1.2	–	Wada et al. [16]
DR1	Δ*pyk*, $PEPC^R$	36.9	4.4	–	
Lysine producer					
P	AK^R	–	–	9.36	Yanase et al.[17]
D2	AK^R, Δ*pyk*	–	–	10.1	
R2	AK^R, $PEPC^R$	–	–	10.8	
DR2	AK^R, Δ*pyk*, $PEPC^R$	–	–	12.9[b]	
DRL2	AK^R, Δ*pyk*, $PEPC^R$, CS^L	–	–	15.7[b]	

$PEPC^R$, desensitized phosphoenolpyruvate carboxylase resulting from the D299N mutation in the *ppc* gene
AK^R, desensitized aspartokinase resulting from the T311I mutation in the *lysC* gene
CS^L, reduced-activity citrate synthase resulting from the S252C mutation in the *gltA* gene
[a]Not applicable
[b]Significant differences ($P < 0.05$) compared with strain P by *t*-test

Strain R1 produced significantly more (21% more) glutamic acid (31.0 g/L) than did ATCC13032 in a jar fermentor culture under biotin-limited conditions in Medium F4 (Table 2), indicating a positive effect of $PEPC^R$ on glutamic acid production. This effect was also investigated in combination with *pyk* deletion [16]. For this purpose, strain DR1, having both a *pyk* deletion and $PEPC^R$, was constructed using strain D1 as the parent. Glutamic acid production by strain DR1 was elevated up to 36.9 g/L (Table 2), which was 44% higher than that by ATCC13032 and significantly higher than those by D1 and R1. Thus, these two mutations were found to exhibit a synergistic effect on glutamic acid production in *C. glutamicum*.

2.3 *Effects of* pyk *Deletion and Phosphoenolpyruvate Carboxylase Desensitization on Lysine Production*

To clarify the effects of *pyk* deletion and $PEPC^R$ on lysine production, these mutations were introduced into lysine-producing *C. glutamicum* singly or in combination [17]. The *C. glutamicum* ATCC13032 mutant having AK^R conferred by T311I amino acid substitution [10] was used as the parent strain (strain P). Strain P produced 9.36 g/L lysine from 100 g/L glucose in a jar fermentor culture. Under

these conditions, while the simple mutant D2 with *pyk* deletion or R2 with $PEPC^R$ (D299N) showed marginally increased lysine yields (10.1 and 10.8 g/L, respectively; Table 2), the mutant strain DR2 having both mutations showed a synergistic increase in lysine production (38% higher, 12.9 g/L; Table 2).

2.4 *Effect of Citrate Synthase Mutation on Lysine Production*

The importance of CS^L in lysine production was previously suggested by Shiio and coworkers [13]. However, no defined CS-defective mutant had been reported until recently, when *C. glutamicum* mutants with CS^L created by a molecular approach were demonstrated to be effective for boosting lysine production [12]. We also identified a single amino acid substitution (S252C) responsible for CS^L in *C. glutamicum* No. 70 [15]. This mutation was then introduced into the lysine-producing mutant strain DR2 having both *pyk* deletion and $PEPC^R$ mutations. The resulting strain, DRL2, showed a greater increase in lysine yield than did strain DR2 (22% higher, 15.7 g/L; Table 2). Thus, the CS^L mutation was confirmed to be effective for lysine production, and these three mutations, *pyk* deletion, $PEPC^R$, and CS^L, were found to enhance lysine yield coordinately in *C. glutamicum* [17].

2.5 *Decreased Phosphoenolpyruvate Carboxykinase Activity Associated with* pyk *Deletion*

In both glutamic acid-producing and lysine-producing strains, alterations of the enzyme activities of the anaplerotic pathway were measured. In all of the *pyk*-deleted strains evaluated, D1, DR1, D2, and DR2, an increase in PEPC and a significant decrease in phosphoenolpyruvate carboxykinase (PEPCK) activities were detected compared with those in the corresponding *pyk* wild-type strains. As an example, changes in enzyme activities in strain D1 during culture for glutamic acid production in Medium F4 under biotin-limited conditions are shown in Table 3. The strain C1, whose *pyk* had been complemented by plasmid, showed similar PEPC and PEPCK activities to those in the wild-type strain, suggesting that the observed enzyme activity changes were caused by the *pyk* deletion. PEPCK activities in strains D2 and DR2 were 17% and 54% of those in the parental strain P and strain R2, respectively [17].

Since the enhanced PEPC activity and reduced PEPCK activity may boost anaplerotic flux from PEP to OAA, the *pyk*-deleted strains seemed to avoid PEP overaccumulation resulting from the absence of the PYK reaction. Aspartic acid formation in the culture medium under biotin-limited conditions (Table 2) also indicated increased OAA availability in the *pyk*-deleted mutants, as aspartic acid is synthesized via a one-step aminotransferase reaction from OAA (Fig. 1); the *pyk*-

Table 3 Activities of enzymes involved in phosphoenolpyruvate/oxaloacetate metabolism and electron transfer to the respiratory chain

Enzyme	Specific activity [nmol min^{-1} (mg protein)$^{-1}$][a]			Ratio (D1/wild-type)
	Wild-type	Strain D1	Strain C1	
Medium F4[b]				
PYK	872 ± 56	<10	1209 ± 220	–[c]
PEPC	143 ± 15	175 ± 1.0	132 ± 3.0	1.22[d]
PEPCK	53.5 ± 4.5	22.6 ± 4.7	52.9 ± 9.0	0.422[d]
MQO	384 ± 55	417 ± 89	303 ± 63	1.09
MDH	638	672	650	1.05
Medium F5[e]				
PYK	931 ± 197	<10	1057 ± 199	–
PEPC	103 ± 7.6	157 ± 7.9	136 ± 33	1.52[d]
PEPCK	186 ± 14	22.5 ± 2.9	156 ± 5.0	0.121[d]
MQO	698 ± 99	260 ± 39	770 ± 75	0.372[d]
MDH	1743 ± 360	1713 ± 162	1684 ± 236	0.983
LldD	61.5 ± 14	35.4 ± 17	53.9 ± 22	0.576
SDH	64.0 ± 6.0	53.7 ± 16	53.8 ± 8.7	0.839
NDH-II	451 ± 35	252 ± 44	467 ± 10	0.559[d]

[a]Values are mean ± SD ($n = 3$) except for MDH ($n = 2$)
[b]Complex medium containing 3 μg/L biotin. Data from a report by Sawada et al. [14]
[c]Not applicable
[d]Significant differences ($P < 0.05$) by *t*-test
[e]Complex medium containing 60 μg/L biotin. Data from a report by Sawada et al. [20]

deleted mutants, strains D1 and DR1, produced 2.3 and 4.4 g/L aspartic acid, respectively, while the wild-type strain produced only 0.44 g/L.

2.6 *Interrelationship Among* pyk *Deletion, Phosphoenolpyruvate Carboxylase Resistant to Feedback Inhibition by Aspartic Acid, and Low-Activity-Level Citrate Synthase*

Notably, CS^L and $PEPC^R$ mutations were simultaneously introduced when a prototrophic revertant, No. 15, was derived from a CS-defective glutamic acid auxotroph, *B. flavum* No. 214, by Shiio and coworkers [18]. Furthermore, the PYK-defective mutation was also simultaneously introduced when an *S*-(2-aminoethyl)-L-cysteine (AEC)-resistant lysine-producing mutant, No. 1-231, was derived from strain No. 15 harboring both CS^L and $PEPC^R$ [1]. These findings suggested causal relationships between these mutations as the probability of two mutations occurring simultaneously is extremely low.

It was speculated that $PEPC^R$ may relieve the stress caused by CS^L, such as the possible accumulation of PEP. In addition, the PYK-defective mutation seemed to be necessary to increase the intracellular lysine concentration by an enhanced OAA

supply to competitively cancel out the toxic effect of a high concentration of AEC, a lysine analogue. PEPC^{R} may also stabilize the effects of PYK-defective mutation by enabling the smooth conversion of PEP into OAA, thus avoiding the accumulation of toxic PEP.

The fact that the previously reported CS^{L} mutants [12] were derived from a strain harboring the P458S mutation in *pyc* (enhanced PC activity, [10]) supported our speculation, as this mutation may relieve the metabolic constraints, for example, the possible accumulation of pyruvic acid, caused by CS^{L}. We also experienced difficulty in introducing the CS^{L} mutation (S252C) into strain P to obtain the simple CS^{L} mutant (unpublished results). The obtained CS^{L} mutant exhibited unstable growth and lysine productivity (lower than or comparable to that of strain P). Although this issue was not examined, it is possible that the CS^{L} was compensated for by the expression of another enzyme possessing CS activity (e.g., methylcitrate synthase) [19], possibly leading to the observed instability. These findings suggest that the CS^{L} mutant strains are stable only under conditions in which the anaplerotic reactions (PEPC or PC) have been enhanced.

2.7 Conclusion

All three mutations focused on by Shiio and coworkers during the 1980s in random mutagenesis studies, *pyk* deletion, PEPC^{R}, and CS^{L}, have been shown to have positive effects on lysine production using modern recombinant DNA techniques as well. The positive effect of *pyk* deletion on lysine production was more evident against a PEPC^{R} background. Under lysine-producing (biotin-sufficient) conditions, PC (Fig. 1), but not PEPC, was regarded as the major anaplerotic enzyme supplying OAA. However, our results also showed the importance of the PEPC reaction as an alternative anaplerotic reaction when its feedback inhibition is deregulated. Thus, our results highlight the potential for *pyk* deletion in combination with PEPC^{R} to enhance the anaplerotic reaction to improve lysine and glutamic acid production in *C. glutamicum*.

3 Enhanced Biomass Production in a *pyk*-Deleted Mutant of *Corynebacterium glutamicum* Cultured Under Biotin-Sufficient Conditions

3.1 Introduction

The previous section described evaluation of the fermentation characteristics of a simple *pyk*-deleted mutant, strain D1, under biotin-limited conditions, which revealed 25% higher glutamic acid yield than that in the wild-type strain

[14]. This section summarizes the results of evaluating strain D1 under biotin-sufficient conditions. Surprisingly, strain D1 showed increased biomass production, which has not been described as a phenotype of *C. glutamicum* mutants during the long history of studies on this species.

3.2 *Enhanced Biomass Production in a* pyk*-Deleted Mutant Under Biotin-Sufficient Conditions*

Enhanced biomass production in a *pyk*-deleted mutant under biotin-sufficient conditions was evaluated in both complex Medium F5 and minimal CGXII medium, each containing 60 μg/L biotin [20]. Interestingly, strain D1 showed a 37% increase in growth in Medium F5 (based on OD_{660}; Fig. 2A) compared with the wild-type ATCC13032. An increased rate of specific glucose consumption (consumed glucose (g) [dry cell weight (g)]$^{-1}$ h^{-1}) was also observed (Fig. 2B) (35% higher than that in the parent). In addition, increased biomass production was observed in the minimal CGXII medium, although the increase (16%, based on OD_{660}) was smaller than that observed in Medium F5. The *pyk*-complemented strain, C1, showed the same growth level and glucose consumption rate as those of ATCC13032 (Figs. 2A, B), indicating that the observed phenotypes had been caused by the *pyk* deletion.

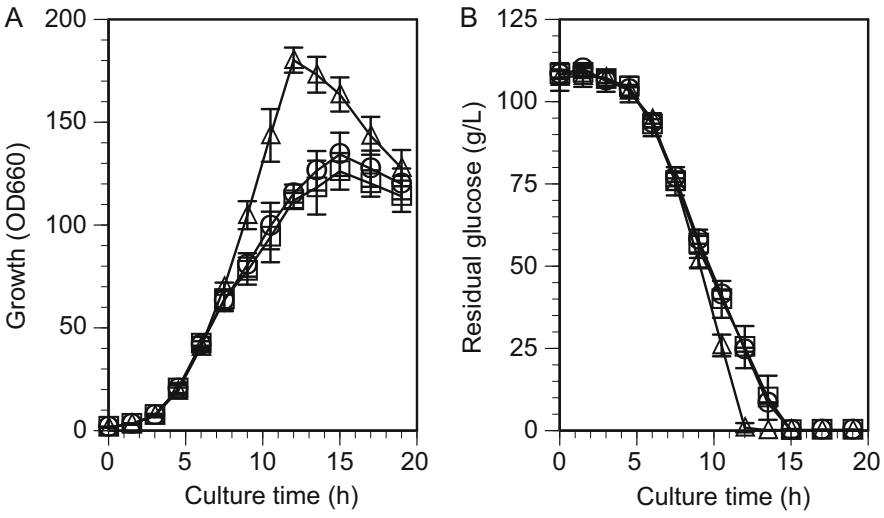

Fig. 2 Profiles of growth and glucose consumption of the wild-type *C. glutamicum* ATCC13032 strain, *pyk*-deleted mutant (D1), and *pyk*-complemented strain (C1) cultured in Medium F5. Reproduction of (**A**) and (**B**) from the report by Sawada et al. [20]. (**A**) Growth and (**B**) glucose consumption. Symbols: *circle*, wild-type strain; *triangle*, D1 strain; *square*, C1 strain. Values are means of three independent experiments. Bars represent the standard deviations

3.3 *Measurements of Anaplerotic Enzyme Activities in a* pyk*-Deleted Mutant*

Both PEPC and PEPCK activities were measured in cells cultured in Medium F5 under biotin-sufficient conditions (Table 3). Strain D1 showed a 52% increase in PEPC activity and an 88% decrease in PEPCK activity compared with ATCC13032, which were similar to the changes observed in Medium F4 under biotin-limited conditions, although the changes were more pronounced in Medium F5 than in Medium F4. These simultaneous changes in PEPC and PEPCK activities seemed to represent an important strategy to avoid PEP overaccumulation caused by *pyk* deletion, irrespective of the biotin concentration. The *pyk*-complemented strain C1 also showed enzyme activities similar to those of the wild-type strain (Table 3).

3.4 *Metabolome Analysis*

Clarification of the mechanism underlying the increased biomass production appeared to be difficult. Eventually, we implemented a metabolome analysis using cells cultured in CGXII medium, which provided valuable information to explain the enhanced growth in the mutant [20]. As shown in Table 4, there was accumulation of many intermediate metabolites located upstream of the PYK reaction, in both the glycolytic pathway and the pentose phosphate pathway, some of which were precursor metabolites for biomass production. On the other hand, the levels of intermediate metabolites located downstream of the PYK reaction, including the TCA cycle metabolites, were decreased. The intermediate concentration ratios of D1 to ATCC13032 tended to increase at 11 h (early stationary phase) compared with those at 6 h (logarithmic growth phase) (Table 4), suggesting increased metabolic distortion toward early stationary phase culture in strain D1. The accumulated precursor metabolites seemed to be converted into biomass, which probably relieved the metabolic distortion in strain D1. Metabolic changes in strain D1 under biotin-sufficient conditions, deduced from the glucose consumption rate, changes in enzyme activity, transcriptional analysis, and metabolome analysis, are summarized in Fig. 3 [20].

3.5 *Excretion of Glycolytic Intermediates in* pyk*-Deleted Lysine Producers*

Notably, previously reported simple *pyk*-deleted lysine producers (Table 1) not only showed lower lysine yields than those of their parents, but also produced glycolytic intermediate-related compounds such as dihydroxyacetone in combination with

Table 4 Ratios of metabolite concentrations in D1 strain cells to those in wild-type strain cells cultured in CGXII medium

Metabolite	Ratio (D6h/W6h)[a]	(D11h/W11h)[b]
Glycolytic pathway		
Glucose 6-phosphate[c]	2.63	2.59
Fructose 6-phosphate[c]	2.28	1.79
Fructose 1,6-diphosphate	1.58	1.41
Dihydroxyacetone phosphate[c]	1.22	1.70
3-Phosphoglyceric acid[c]	1.93	3.27
Phosphoenolpyruvate[c]	2.11	5.59
Pyruvic acid[c]	0.899	0.214
TCA cycle		
Acetyl CoA[c]	0.663	0.542
Citric acid	0.523	0.676
2-Oxoglutaric acid[c]	1.17	0.816
Succinic acid	0.708	0.522
Fumaric acid	1.03	0.719
Malic acid	0.844	0.656
Pentose phosphate pathway		
6-Phosphogluconic acid	1.83	8.23
Ribulose 5-phosphate	1.79	4.32
Ribose 5-phosphate[c]	1.55	2.57
Sedoheptulose 7-phosphate	2.51	6.31

The data are from Table 5 in the report by Sawada et al. [20]

[a]Ratio of metabolite concentrations in strain D1 cultured for 6 h to those in the wild-type strain cultured for 6 h

[b]Ratio of metabolite concentrations in strain D1 cultured for 11 h to those in the wild-type strain cultured for 11 h

[c]Precursor metabolites essential for biomass synthesis

either glycerol or glyceraldehyde [7–9]. These C3 metabolites may be derived from dihydroxyacetone phosphate or glyceraldehyde phosphate. For example, *pyk*-gene-disrupted *Corynebacterium lactofermentum* L124 produced both 3.5 g/L dihydroxyacetone and 14.7 g/L glyceraldehyde along with 15 g/L lysine, while its parent strain *C. lactofermentum* ATCC21799 produced 26.3 g/L lysine, without these C3 metabolites arising as by-products [7].

Under these conditions, our metabolome analysis strongly suggested that, in simple *pyk*-deleted lysine-producing mutants, the accumulation of glycolytic intermediates induced C3 metabolites as by-products, which reduced the lysine yield. On the other hand, in lysine producers such as strain DR2, having both *pyk* deletion and PEPC^{R}, glycolytic intermediates seemed to be smoothly converted to OAA by the deregulated PEPC, leading to improved lysine production without C3 metabolites arising as by-products.

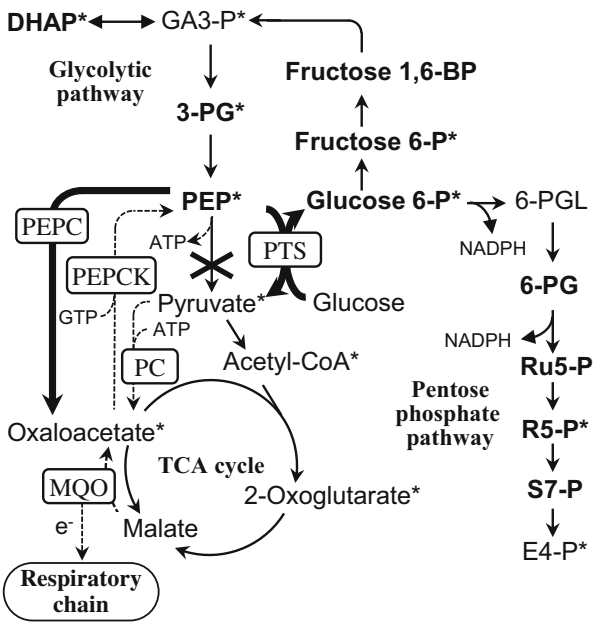

Fig. 3 Schematic model of carbon flux in *C. glutamicum* strain D1. The cross represents *pyk* deletion. Reproduction of Fig. 3 from the report by Sawada et al. [20]. The *thick arrow line* indicates increased flux and the *dotted arrow line* decreased flux, as deduced from enzyme activity measurements, transcriptional analysis, or rate analysis. Metabolites that increased in abundance as detected by metabolome analysis of strain D1 are shown in boldface. Asterisk indicates a precursor metabolite for biomass synthesis. *Glucose 6-P* glucose 6-phosphate, *PTS, PEP* carbohydrate phosphotransferase system, *fructose 6-P* fructose 6-phosphate, *fructose1,6-BP* fructose1,6-bisphosphate, *GA3-P* glyceraldehyde 3-phosphate, *DHAP* dihydroxyacetone phosphate, *3-PG* glycerate 3-phosphate, *6-PGL* 6-phosphogluconolactone, *6-PG* 6-phosphogluconate, *Ru5-P* ribulose 5-phosphate, *R5-P* ribose 5-phosphate, *S7-P* sedoheptulose 7-phosphate, *E4-P* erythrose 4-phosphate. Other abbreviations are the same as shown in the legend of Fig. 1

Table 5 Fold changes in the activities of MDH, MQO, and respiration in ATPase and *pyk* mutants of *C. glutamicum* compared with those in their respective parental strain

Fold change mutant strain/parent strain				
	C. glutamicum A-1	*C. glutamicum* D1		*C. glutamicum* D2
	ATPase-defect	Δ*pyk*		AK^R, Δ*pyk*
	Medium G3[a]	Medium F4	Medium F5	Medium L1[b]
MDH	2.0 ↑	1.05 →	0.98 →	0.96 →
MQO	2.6 ↑	1.09 →	0.37 ↓	0.38 ↓
Respiration	1.4 ↑	1.00 →	0.43↓	0.65↓

Symbols: upward arrow, increased; rightward arrow, no change; down ward arrow, decreased
[a]Semisynthetic medium containing 60 μg/L biotin [22]
[b]Lysine production medium containing 300 μg/L biotin [17]

4 Alterations of Malate:Quinone Oxidoreductase Activity in a *pyk*-Deleted Mutant and Elucidation of its Physiological Roles

4.1 Introduction

Malate:quinone oxidoreductase (MQO) is a unique TCA cycle enzyme that catalyzes the conversion of malate to OAA with concomitant electron transfer to menaquinone in the respiratory chain [21]. During the characterization of *pyk*-deleted mutants, we found another interesting role of MQO in the regulation of carbon metabolism in *C. glutamicum*, which is described in this section.

4.2 Malate:Quinone Oxidoreductase as the Main Site of Entry for Electrons into the Respiratory Chain in Corynebacterium glutamicum

In *C. glutamicum*, it has been reported that MQO constitutes an NADH reoxidation system in coupling with malate dehydrogenase (MDH) that catalyzes the reverse reaction with concomitant oxidation of NADH. In our previous study [22], measurements of the activities of known enzymes involved in NADH reoxidation other than MQO and MDH, quinone-dependent lactate dehydrogenase (LldD) and lactate dehydrogenase (LdhA), both constituting a similar coupling system to MQO/MDH, and NADH dehydrogenase-II (NDH-II), identified the MQO/MDH coupling system as a major gate of electron transfer from NADH to the respiratory chain, judging from the far higher MQO activity than those of LldD and NDH-II. In line with these observations, an H^+-ATPase-defective mutant of *C. glutamicum*, strain A-1, which showed enhanced glucose consumption and respiration, exhibited elevations in both MQO/MDH activities and their gene expression levels compared with those in the parent strain, ATCC13032 ([22]; Table 5).

4.3 Malate:Quinone Oxidoreductase as a Controller of the Respiration Rate in Corynebacterium glutamicum

The finding that MQO is involved in OAA metabolism prompted us to measure MQO activity in the simple *pyk*-deleted mutant, strain D1, during culture under biotin-sufficient conditions in Medium F5 [20]. In terms of the results, a dramatic decrease in MQO activity down to 37% of that in the parent ATCC13032 was found, with a concomitant decrease in the respiration rate to 43% of that in the

parent (Tables 3 and 5). These changes recovered to the parental levels in the *pyk*-complemented strain C1 (Table 3). The decreased MQO activity was regulated at the transcriptional level [20]. Similar simultaneous decreases in both MQO activity and the respiration rate were also observed in the *pyk*-deleted lysine producer strain D2 (38% MQO activity/65% respiration rate compared with the parent strain P, Table 5; [17]). In both cases, no significant change in activity was observed in MDH (Tables 3 and 5). These positive correlations between MQO activities and respiration rates observed in both of the *pyk*-deleted mutants (strains D1 and D2) and the aforementioned H^+-ATPase-defective mutant (strain A1) led us to conclude that MQO may be involved in respiration control in *C. glutamicum*. Measurements of respiration-related enzymes, namely MQO, LldD, SDH, and NDH-II, in the wild-type ATCC13032 cultured in Medium F5 again confirmed MQO as the major enzyme contributing to respiration (Table 3).

4.4 Malate:Quinone Oxidoreductase as a Fine-tuner of Oxaloacetic Acid Concentration

Apart from the roles played by MQO in respiration control in *C. glutamicum*, its possible role in carbon metabolism as a TCA cycle enzyme needs to be considered. Our study revealed that, in contrast to the case under biotin-sufficient conditions, such coordinated decreases in MQO activity and respiration rate were not observed in strain D1 when cultured under biotin-limited conditions in Medium F4 during glutamic acid production [20]. In *pyk*-deleted mutants, strains D1 and D2, cultured under biotin-sufficient conditions, development of an excessive OAA supply was apparent, which probably led to the decreased MQO activity to relieve OAA overaccumulation (Fig. 1). However, under biotin-limited conditions in Medium F4, OAA overaccumulation did not seem to take place as TCA cycle intermediates including OAA were consumed as precursors of glutamic acid biosynthesis, thereby probably rendering a decrease in MQO activity unnecessary. These observations led us to propose an additional role of MQO in carbon metabolism as a modulator or safety valve to fine-tune the OAA concentration, as well as its role in controlling the respiration rate (redox balance) in *C. glutamicum* [20]. The regulation of MQO gene expression in response to these metabolic constraints needs to be clarified in the future to obtain a clearer understanding of the roles of MQO.

5 Conclusions

Our studies on *pyk*-deleted mutants clarified the conditions under which the PEPC reaction is a useful anaplerotic reaction to supply OAA for lysine and glutamic acid production in *C. glutamicum*. Furthermore, detailed physiological studies suggested

MQO to be important in controlling both carbon flow at the OAA node and respiration rate/redox balance. These findings may contribute to the rational use of this bacterium in fermentation industries.

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Exporters for Production of Amino Acids and Other Small Molecules

Lothar Eggeling

Abstract Microbes are talented catalysts to synthesize valuable small molecules in their cytosol. However, to make full use of their skills – and that of metabolic engineers – the export of intracellularly synthesized molecules to the culture medium has to be considered. This step is as essential as is each step for the synthesis of the favorite molecule of the metabolic engineer, but is frequently not taken into account. To export small molecules via the microbial cell envelope, a range of different types of carrier proteins is recognized to be involved, which are primary active carriers, secondary active carriers, or proteins increasing diffusion. Relevant export may require just one carrier as is the case with L-lysine export by *Corynebacterium glutamicum* or involve up to four carriers as known for L-cysteine excretion by *Escherichia coli*. Meanwhile carriers for a number of small molecules of biotechnological interest are recognized, like for production of peptides, nucleosides, diamines, organic acids, or biofuels. In addition to carriers involved in amino acid excretion, such carriers and their impact on product formation are described, as well as the relatedness of export carriers which may serve as a hint to identify further carriers required to improve product formation by engineering export.

Keywords Amino acids, Biofuels, Diamines, Excretion, Export, Nucleosides, Organic acids, Peptides

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Contents

1 Introduction

It is standard practice to perform pathway engineering in order to increase product formation. However, this is much less frequently the case in engineering the export process. It is now accepted that the export of the product from the cell is just as important as its synthesis in the cell, but nevertheless there are as yet few examples of the targeted engineering of export processes. This is due, among other things, to the fact that with living cells (1) the quantification of export parameters such as diffusion and active export is laborious, (2) export takes place via various cell envelope components, (3) the exporter for a specific product may be unknown, and (4) it may not even exist. Selectively increasing export performance is therefore relatively laborious especially since it is known that the simple overexpression of membrane proteins, as takes place with plasmid-encoded carrier genes, can be detrimental for the cell [1].

Extensive information in particular on the export of small and biotechnologically relevant molecules is available for the amino acids. Reviews have already been published on the subject [2–4]. A good overview of the mechanism of all carriers present in *E. coli*, respectively, *C. glutamicum*, as well as their known or derived functions, can be found [5, 6]. A list comparing all the transporters of *C. glutamicum* and *Corynebacterium efficiens* as deduced from genome sequences is also available [7]. The present chapter focuses on the export of a specific amino acid or export of another small molecule or a specific transporter family with

functionally identified examples of carriers, depending on the information available. First of all, a very brief overview of transport proteins and how they can be accessed will be given.

2 Overview on Exporters and Their Isolation

The various proteins involved in the transport of amino acids and other small solutes can be categorized on the basis of their structural, phylogenetic, and functional properties. Extensive information can be found in databases and in recent monographs [8–10]. Figure 1 gives a schematic overview of the efflux of the proteins involved. Most amino acid export carriers are secondary active carriers. They use the electrochemical H^+ or Na^+ potential across the cytoplasmic membrane as a driving force, whereas primary active carriers use the free energy of ATP binding and hydrolysis to catalyze export [12]. Only one primary active carrier is known to be involved in amino acid export which is CydDC of *E. coli* exporting L-cysteine to the periplasmic space. Interestingly, most of the exporters improving fuel tolerance are primary active carriers (Table 1). A special case is tripartite carriers, where the carrier is linked to a third protein anchored in the outer

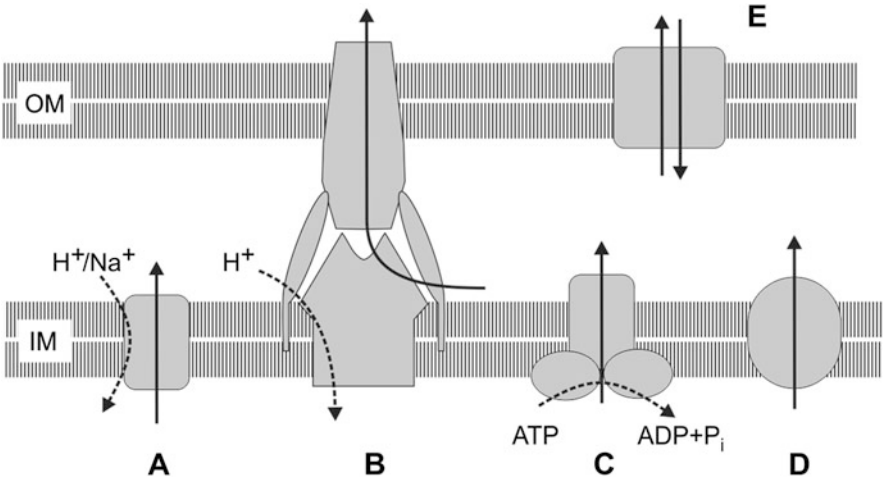

Fig. 1 A gallery of proteins involved in small molecule export. *E. coli* and also *C. glutamicum* possess an inner membrane (IM) and an outer membrane (OM) [11]. A large diversity of solute exporters use the H^+ or Na^+ potential as the solute driving force (*A*, *B*). Tripartite carriers bridge the inner and the outer membrane (*B*). So far they are only known from Gram-negative bacteria, and they export solutes present in the outer leaflet of the IM or present in the periplasmic space. ABC proteins use ATP to transport solutes across the inner membrane (*C*). Mechanosensitive channels operate in an energy-independent manner, usually in response to mechanical stress (*D*). This type of export is the major reason for the L-glutamate efflux of *C. glutamicum* and is not known to operate for the export of other amino acids, fuels, or antibiotics. Porins are located in the outer membrane; these are channels and enable the passive diffusion of solutes (*E*)

Table 1 Overview on genes relevant for small molecule export

Gene	Known function (reference)	Origin	Category
lysE	Export of basic amino [13–15]	*C. glutamicum*	LysE
argO	Export of Arg and canavanine [16]	*E. coli*	LysE
msiA	Export of canavanine [17]	*Mesorhizobium tianshanense*	LysE
rhtA	Export of Thr and homoserine [18, 19]. Export of 5-aminolevulinic acid [20]	*E. coli*	DMT
rhtB	Export of Thr [21]. Resistance toward Thr, homoserine, homoserine lactone [22, 23]	*E. coli*	LysE
rhtC	Resistance toward Thr [22, 23]	*E. coli*	LysE
yeaS (leuE)	Export of Leu [24]. Resistance toward Thr, homoserine, homoserine lactone [22]	*E. coli*	LysE
thrE	Export of Thr, Ser [25, 26]	*C. glutamicum*	ThrE
yddg	Export of aromatic amino acids [27]	*E. coli*	DMT
ydeD (eamA)	Export of O-acetylserine and Cys [28]. Export of dipeptides [29]	*E. coli*	DMT
bcr	Export of Cys [30]. Export of dipeptides [29]	*E. coli*	MFS
yfiK (eamB)	Export of O-acetylserine and Cys [31]. Resistance against Thr [22]	*E. coli*	LysE
ybjE	Export of L-lysine [32]	*E. coli*	CaCA
cydDC	Export of Cys [33]	*E. coli*	ABC
yijE	Resistance against cystine [34]	*E. coli*	DMT
tolC	Supports Cys accumulation [35]	*E. coli*	OMF
brnFE	Export of Met, Leu, Ile, Val [36, 37]	*C. glutamicum*	LIV-E
ygaZH	Export of Ile [38]	*E. coli*	LIV-E
leuE	Export of Leu, Val [39]	*Gluconacetobacter europaeus*	LIV-E
ygaW (alaE)	Export of Ala [40]	*E. coli*	AlaE
ytfF	Export of Ala [40]	*E. coli*	LysE
mscCG (YggB)	Efflux of Glu [41, 42]	*C. glutamicum*	MscS
cadE	Export of cadaverine [43]	*C. glutamicum*	MFS
sucE	Export of succinate [9, 44]	*C. glutamicum*	AAEx
pbuE (ydhL)	Export of purine base and purine nucleosides [45]	*B. subtilis*	MFS
YicM (Nepl)	Export of purine nucleosides [46]	*E. coli*	MFS
cepA	Resistance against purine analogues [47]	*C. glutamicum*	MFS
acrAB	Improvement of limonene accumulation [48]	*Alcanivorax borkumensis*	ABC
algE	Export of neutral lipids [49]	*Alcanivorax borkumensis*	OM
setA	Export of sugars [50]	*E. coli*	MFS
FpOAR	Export of oxalate [51]	*Fomitopsis palustris*	DedA

(continued)

Table 1 (continued)

Gene	Known function (reference)	Origin	Category
Pdr12p	Export of aromatic and branched-chain organic acids [52]	*S. cerevisiae*	ABC
mdlB	Improvement of isopentenol accumulation [53]	*E. coli*	ABC
msbA	Improvement of canthaxanthin accumulation [54]	*E. coli*	ABC
Snq2p	Improvement of decane, undecane accumulation [55]	*S. cerevisiae*	ABC
Pdr5p	Improvement of decane, undecane accumulation [55]	*S. cerevisiae*	ABC
ABC2	Increased tolerance against decane, undecane [56]	*Yarrowia lipolytica*	ABC
ABC3	Increased tolerance against decane, undecane [56]	*Yarrowia lipolytica*	ABC
ADP1	Increased tolerance against ethanol [57]	*S. cerevisiae*	ABC

The categorization was done according to www.tcdb.org/
The abbreviations represent the following families, respectively, superfamilies: *LysE* L-lysine exporter, *DMT* drug/metabolite transporter, *ThrE* threonine/serine exporter, *MFS* major facilitator, *ABC* ATP-binding cassette, *OMF* outer membrane factor (porin), *LIV-E* branched-chain amino acid exporter, *AlaE* L-alanine exporter, *MscS* small conductance mechanosensitive ion channel, *AAEx* aspartate:alanine exchanger

membrane via an auxiliary protein. In this manner, an exit duct is formed releasing the solute directly into the surroundings of the cell. Components of tripartite carriers are of relevance for L-cysteine formation and fuel tolerance. Mechanosensitive channels are energy independent. They are located in the cytoplasmic membrane and open in cases of emergency for the fast release of small internal solutes. The recently discovered significance of a mechanosensitive channel for L-glutamate export in *C. glutamicum* was a great surprise. The pore-forming outer membrane proteins are also energy independent [58], and they may play a role in small solute formation, too (see below).

The genes involved in export can be isolated in various ways. It is often necessary to have more extensive knowledge or to perform preceding work in order to enable increased export of the respective metabolite. Key ways and means for carrier identification include the following:

- The use of export-negative mutants and isolation of complementing fragments, as was done for LysE of *C. glutamicum* [13, 59].
- Isolation of mutants with increased sensitivity to peptides containing the amino acid of interest and their analysis or complementation. Examples are BrnFE [36] of *C. glutamicum* and AlaE of *E. coli* [60]. A special case is the use of the Keio collection which was advantageously employed to identify mutants with hypersensitivity to L-cysteine due to the absence of TolC [35].
- Increased resistance to the amino acid or its analogue. *rhtA23* of *E. coli* was identified in this manner [61].

- Screening of chromosomal fragments for increased product accumulation. This was done for *ydeD* of *E. coli* [28].
- Identification of genes with increased transcription in response to increased metabolite concentration, as was used to isolate YddG of *E. coli* [62], the diaminopentane exporter [43], and SucE of *C. glutamicum* [44].
- Screening panels of carrier genes derived from bioinformatic approaches for increased production. Examples are screening for cysteine exporters [30] or biofuel exporters [48].

3 L-Lysine Export by *C. glutamicum*

L-Lysine is one of the amino acids with the strongest growth rate of about 5–7% per year. Currently, 2.2×10^6 tonnes are produced each year, most of which is produced using *C. glutamicum* [63]. Pioneering work by Krämer showed that an understanding of the transport processes is absolutely indispensable for product formation [64]. On this basis, it proved possible to clone the L-lysine exporter of *C. glutamicum* [13]. This very first exporter for a biotechnologically relevant amino acid showed that L-lysine formation with *C. glutamicum* was completely dependent on the carrier. This motivated further work on amino acid export carriers, especially at the Ajinomoto-Genetika Research Institute in Moscow. The transcription of *lysE* is controlled by LysG which displays the characteristic structural features of an autoregulatory transcriptional regulator from the large group of LysR-type transcriptional regulators (LTTR) [14]. The L-lysine export process is very efficient. Two excellent L-lysine producers of *C. glutamicum*, strain MH20-22B [65] and strain B-6 [66], both obtained by a series of undirected mutagenesis do not have a mutation in *lysE* nor in *lysG*. A second *lysE* copy in a molecularly defined strain leads to an increased L-lysine export rate under certain experimental conditions, but does not yield increased L-lysine accumulation in fermentations [67]. The export process requires the transcription of *lysE*. This only takes place at an increased cytosolic concentration of about 20 mM L-lysine [14]. As already mentioned, the transcription is controlled by LysG, whereby LysG cannot only interact with L-lysine as a coinducer but also with the basic amino acids L-arginine, L-citrulline, and L-histidine. However, L-citrulline and L-histidine do not serve as substrates for LysE. L-Lysine and L-arginine are exported at comparable rates of about 0.75 nmol/min/(mg dry wt) [14].

The exporter LysE is apparently promiscuous with respect to the chirality of lysine. This is indicated by the fact that after expression of the racemase ArgR from *Pseudomonas taetrolens*, *C. glutamicum* excretes D- and L-lysine. However, no excretion takes place after the deletion of *lysE*, but rather the internal accumulation of the racemate [68]. In contrast, diaminopimelate [14] and diaminopentane [43] are not exported by LysE.

4 LysE Use in *Methylobacillus*

Methanol can be obtained from carbon dioxide and has the potential to be used as a carbon and energy source in biotechnological processes. It represents an alternative to sugar utilization since the largest contribution to the variable costs in fermentation-based large-scale amino acid processes is the substrate. It has been possible to engineer the nonmethylotrophic and L-lysine-excreting *C. glutamicum* toward the utilization of methanol as an auxiliary carbon source in a sugar-based medium, but the engineered bacterium is still unable to grow with methanol as sole carbon and energy source [69]. On the other hand, *Methylophilus methylotrophus* is able to grow rapidly on methanol and with a good yield [70]. However, *M. methylotrophus* lacks a specific export system for L-lysine [71]. Initial attempts to introduce LysE of *C. glutamicum* into *M. methylotrophus* failed, and this was found to be due to *lysE* instabilities. However, one particular *lysE-24* mutation was obtained which resulted in a roughly tenfold increase in L-lysine accumulation with *M. methylotrophus* [15]. With *lysE-24* together with an L-lysine biosynthesis gene from *E. coli* (*dapA24*), up to 11.3 g/L L-lysine was achieved in fermentations, with an even further increase when the Entner–Doudoroff pathway was established in addition to the ribulose monophosphate pathway [70]. Also with *Methylobacillus glycogenes*, export of L-lysine was obtained with *lysE-24* illustrating the suitability of the export carrier of *C. glutamicum* to overcome export limitations [15]. The *lysE-24* mutation is a very specific one. It is a point mutation in the long cytosolic loop of the carrier connecting the two halves of the protein between helix 3 and 4 [15, 72]. The mutation results in a chain termination, but the evidence suggests that two separate polypeptides are synthesized due to reinitiation of translation utilizing an existing start codon beyond the mutation site. The loop region itself is not of relevance for export. The reason is that individual deletions in the long cytosolic loop of 52 amino acyl residues, irrespective of where they are located, only weakly influence the export rate in *C. glutamicum* [73] (Haier B., Massow M., Eggeling L., Krämer R, unpublished results). This also agrees with the notion that orthologous LysE proteins exist without a long loop region, as is, for instance, the case with ArgO of *E. coli*.

5 Export by Members of the LysE Family and LysE Superfamily

LysE is the paradigm of the LysE family of export carriers. In fact, it is the first representative of a larger group of transporters categorized as the LysE superfamily [74]. All members are small proteins consisting of about 220–250 amino acyl residues. By analogy with other small transporter proteins, and due to known translocator structures, LysE superfamily members probably have six transmembrane-spanning helices and function as a dimer. Constituent members

of the LysE superfamily are the LysE, the RhtB, and the CadD family [74]. The functionally characterized members of the individual families export small solutes. This confirms the notion that members of a single transporter family frequently catalyze the transport of structurally related types of compounds (i.e., amino acids or sugars) and, moreover, function with a strongly preferential polarity of the transport direction (i.e., outward or inward) [10]. Members of the CadD family export cadmium and possibly quaternary amines [74, 75]. Members of the RhtB family relevant for amino acid export are RhtB and RhtC, as well as LeuE and YfiK which are treated further below (see also Table 1).

A functionally characterized member of the LysE family is ArgO of *E. coli* [16]. Similar to LysE of *C. glutamicum*, *argO* is controlled by an LTTR, called ArgP. ArgP can interact with L-arginine or L-lysine, but a final promoter clearance step enables *argO* transcription only by L-arginine [76]. The carrier exports L-arginine as well as canavanine. The carrier YbjE (LysO) mediates export of L-lysine [32]. Canavanine is a structural analogue of L-arginine exuded by some legumes such as lupins where this toxic non-proteinogenic amino acid serves as an anti-herbivore defense. Canavanine is also toxic for microorganisms. It is therefore probably not surprising that in *Mesorhizobium tianshanense*, a rhizobium that forms nodules on licorice (*Glycyrrhiza uralensis*) plants, genes encoding a LysE carrier and an adjacent LTTR are present, here called MsiA and MsiR [17]. MsiA serves as a canavanine exporter that is indispensable for canavanine resistance in *M. tianshanense* and is activated by MsiR together with canavanine. Also in other rhizobial species, MsiA homologues are present whose expression is induced by canavanine and is critical for canavanine resistance. There is functional evidence of a further LysE-type transporter, which is AttX of the phytopathogen *Rhodococcus fascians*. AttX is present in the *att* locus, and the entire locus is relevant for synthesis and virulence due to an unidentified compound made by the *att* genes [77]. Since the *attA*, *attB*, and *attH* biosynthesis genes share identities with L-arginine biosynthesis genes, a possible scenario is that the LysE homologue AttX excretes a structural analogue to arginine as the virulence-inducing factor.

6 L-Threonine Export by *E. coli*

The global demand for L-threonine has experienced double-digit growth rates since the commercial application took off in the mid-1990s. Excellent L-threonine producers from *E. coli* have been developed using undirected mutagenesis and consecutive screening steps [61]. One of these strains was found to be sensitive to 5 mg/mL L-threonine on minimal medium M9, but spontaneous mutants resistant to this inhibitory concentration were observed. The characterization of one of them, strain VNIIgenetika-472T23, resulted in the isolation of *rhtA*23 [18]. The exporter gene *rhtA*23 in *E. coli* VNIIgenetika-472T23 carries an "*A*" for "*G*" substitution in the promoter region, which causes a tenfold increase in the expression of *rhtA*23 as compared to *rhtA*. The *rhtA* gene confers resistance to L-threonine, L-homoserine,

and further selected amino acids as well as amino acid analogues. In an L-threonine producer of *E. coli*, inactivation of *rhtA* results in a slightly decreased export rate, but *rhtA*23 overexpression increases the L-threonine export rate about twofold. In fermenters, *rhtA*23 overexpression resulted in 36.3 g/L L-threonine accumulation, whereas 18.4 g/L was obtained with the control.

In addition to *rhtA*, *E. coli* also possesses the *rhtB*, *rhtC*, and *yeaS* genes involved in L-threonine export [21, 22, 78]. Data for comparing the capacity of these transporters are scarce, but *rhtA* and *rhtC* can be regarded as most effective [78]. The deletion of *rhtB* together with *rhtC* resulted in a slightly reduced maximal specific excretion rate in L-threonine production [23]. A global gene expression analysis of a defined L-threonine producer revealed increased expression of *rhtC* [79]. The subsequent construction of a strain with amplified *rhtC* produced a 50.2% higher L-threonine concentration than that obtained with the control, thus reinforcing the relevance of L-threonine export for production. The additional amplification of the *rhtA* and *rhtB* genes only resulted in a slightly further increased L-threonine formation. Patent literature discloses further transport genes increasing L-threonine export such as *yedA*, *ychE*, *ygaZH*, and *eamB* without detailed evaluation of their relevance [78]. As far as is known, most of the abovementioned transporters do not only accept L-threonine as substrate but also other metabolites, often with a higher affinity than L-threonine. In this way, *rhtB* provides primary resistance to L-homoserine, L-homoserine lactone, and further amino acid derivatives (Table 1). With *rhtB* overexpressed, an L-homoserine producer accumulated 10.6 g/L L-homoserine, whereas without *rhtB* present, only 0.35 g/L was found, and the increased export was accompanied by a reduced intracellular L-homoserine concentration [21]. With *rhtA* overexpressed both L-threonine and L-homoserine export is improved. Whereas *rhtA23* enabled a twofold increase in threonine accumulation from 18.4 to 36.3 g/L, the effect with homoserine accumulation was much more dramatic with an increase from 9.0 to 56.0 g/L [18]. The unspecificity of the transporters can lead to the export of undesirable by-products, which is detrimental for the purification and the financial balance of the production process.

7 L-Threonine Export by *C. glutamicum*

L-Threonine is required as a feed additive and is currently added in the form of a crystalline product made by *E. coli*. An economic advantage would be usage of the entire concentrated fermentation broth as a feed additive, as is the case with Biolys, which contains L-lysine together with the biomass of *C. glutamicum* [63]. It therefore almost goes without saying that there have been a number of attempts to use the excellent biosynthetic capacities of *C. glutamicum* for L-threonine production as well. However, strain constructions done by pathway engineering were not successful due to plasmid instabilities [80, 81], which were ultimately found to be caused by limiting export activity [82]. Driven by these findings, the exporter gene *thrE* was isolated from *C. glutamicum* [25]. However, upon overexpression of *thrE*,

and further engineering of the cellular metabolism [83], only limited success with model strains was observed. This shows that ThrE is possibly not natively designed to export L-threonine. Due to its scarce but widespread occurrence, it is regarded as an ancient genomic relict using a still unknown compound as substrate [26].

In a different approach, exporters of *E. coli* were assayed for their use in *C. glutamicum* [84]. Among RhtA, RhtB, RhtC, and YeaS which were assayed, RhtA and RhtC proved to be very effective with respect to extracellular L-threonine accumulation (Fig. 2). RhtA in *C. glutamicum* is unspecific since together with L-threonine it also excretes L-homoserine, and this reflects the situation in *E. coli* [18]. However, with RhtC only L-threonine accumulation was observed. In addition, the use of threonine-tripeptide added extracellularly to the wild type resulted in intracellular accumulation of 140 mM L-threonine, but upon *rhtC* overexpression, under otherwise identical conditions, only an accumulation of 11 mM L-threonine was found. This is a strong indication that RhtC was not yet saturated with substrate, meaning that intracellular L-threonine availability was limiting and providing further room for engineering the central metabolism. Due to *rhtC* expression, the maximal excretion rate of L-threonine in a small-scale fermentation was 11.2 nmol min^{-1} mg (dry weight)$^{-1}$ as compared to 2.3 nmol min^{-1} mg (dry weight)$^{-1}$ obtained without *rhtC* expression. The examples of RhtC use in *C. glutamicum*, LysE use in *M. methylotrophus*, as well as use of the different biofuel efflux pumps (see below) provide confidence in the applicability of exporters in heterologous hosts once an export limitation is recognized.

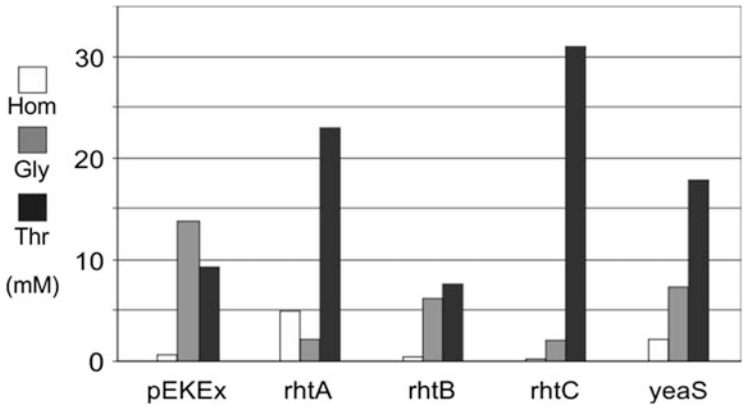

Fig. 2 Production of L-threonine by *C. glutamicum* due to heterologous exporters from *E. coli*. The background strain contained empty vector pEKEx and accumulated mainly glycine which is the degradation product of L-threonine [83]. The genes *rhtA*, *rhtB*, *rhtC*, and *yeaS* were individually overexpressed in the same strain using pEKEx. Overexpression of *rhtA* resulted in improved L-threonine accumulation but in addition also in a considerable accumulation of L-homoserine, whereas with overexpressed *rhtC* almost exclusively L-threonine was formed [84]

8 Export by Members of the RhtA Family

RhtA of *E. coli* is a membrane protein with ten transmembrane-spanning helices. It is the paradigm of the RhtA family which is part of the large drug/metabolite transporter (DMT) superfamily [21]. In addition to L-threonine and L-homoserine export, RhtA of *E. coli* also participates in the export of 5-aminolevulinic acid [20]. This five-carbon amino acid is a native intermediate of tetrapyrrole synthesis and has attracted attention for potential application in specific tumor therapies [85]. After engineering of the biosynthesis pathway and expression of *rhtA*, the 5-aminolevulinic acid accumulation in *E. coli* increased from 1.95 to 2.89 g L^{-1} enabling, under optimized conditions, the accumulation of 4.13 g L^{-1} 5-aminolevulinic acid. *E. coli* contains at least ten paralogues of RhtA [18]. Among them is YdeD catalyzing L-cysteine export [28] and Yddg catalyzing the export of aromatic amino acids [27]. Paralogues of RhtA are present in other bacteria, such as PecM in *Erwinia chrysanthemi* involved in the efflux of the blue pigment indigoidine [86]. In addition, in a number of plant- and insect-associated bacteria, such as *Bacillus thuringiensis*, *Pseudomonas syringae*, and *Pseudomonas savastanoi*, small operons are present with genes encoding an RhtA efflux carrier [87], together with dioxygenases hydroxylating free L-isoleucine or L-leucine [88]. This scenario leads to the assumption that peptide-like substances are synthesized and exported, although these substances are as yet unknown.

9 L-Tryptophan Export by *E. coli*

The *E. coli* exporter for aromatic amino acids, YddG, was identified via homology comparisons with drug/metabolite exporters [27]. The carrier is expressed at a low level, but is induced twofold under salt stress conditions or at an elevated extracellular L-phenylalanine concentration [62]. Its overexpression provides resistance to inhibiting concentrations of L-phenylalanine and the aromatic amino acid analogues, D,L-p-fluorophenylalanine, D,L-o-fluorophenylalanine, and D,L-5-fluorotryptophan [27]. Overexpression of *yddG* in model strains resulted in a 1.5- to 3-fold increase in L-tyrosine, L-phenylalanine, and L-tryptophan accumulation. With a production strain and *yddG* overexpressed, 48.7 g L^{-1} L-tryptophan was obtained, which represented an increase of 15.9% as compared to the parental strain [89].

Besides excretion also uptake may influence the final accumulation of the product, since the total flux over the membrane is made up of active export, active import, and diffusion [5]. The relevance of the individual components varies from amino acid to amino acid and from the physiology and genetic regulation of the cell. To obtain good amino acid production, also reducing import of the amino acids might improve the performance of strains and their accumulation properties. This finding is underpinned by the observation that the presence of multicopies of *aroP*,

responsible for the aromatic amino acid uptake, in an L-tryptophan-producing *C. glutamicum* strain, resulted in a drastic decrease in L-tryptophan formation [90]. Mutants of *C. glutamicum* that were impaired in their uptake of L-tyrosine and L-phenylalanine were shown to accumulate 10–20% more L-tryptophan than their parent in fed-batch fermentations [91]. The same strategy was applied to an *E. coli* model strain producing 1.84 g L^{-1} L-tryptophan [92]. Deletion of the aromatic amino acid importer gene *tnaB* resulted in an increased accumulation of 2.05 g L^{-1} L-tryptophan and the additional deletion of *aroP* in that of 2.44 g L^{-1}. The relevance of reduced import is also known for L-threonine accumulation with *E. coli*. A classical producer strain was found to be impaired in its L-threonine uptake [93], and an increase of 15.6% in L-threonine accumulation was observed when the L-threonine uptake system *tdcC* was inactivated in a well-defined L-threonine producer [79].

10 L-Cysteine Export by *E. coli*

The major proportion of L-cysteine is still produced from hair hydrolysate. However, this process is being replaced by synthesis with *E. coli*. This increases acceptance in the pharmaceutical and food sector and also makes it possible for the product to be used in kosher and halal applications. At least four membrane-embedded exporters as well as one outer membrane protein contribute to the L-cysteine efflux in *E. coli*. The exporters YdeD [28] and YfiK [31] both enable increased accumulation of extracellular L-cysteine and the intermediate O-acetylserine. YdeD also transports L-asparagine and L-glutamine to some extent and probably also 2-methyl-2,4-thiazolidinedicarboxylic acid which is a nonenzymatically formed condensation product of cysteine and pyruvic acid. Both YdeD and YfiK are, furthermore, similarly effective in reducing the growth inhibition due to externally added O-acetylserine, but they differ slightly with respect to their cellular characteristics, such as their growth-phase-dependent activity. The unspecificity of YdeD was also used as a basis for making an O-acetyl-L-serine accumulating strain. With such a strain, accumulation of 9 g/L O-acetyl-L-serine was achieved, which was then used in enzymatic conversions to produce an array of unnatural sulfur-containing L-amino acids of interest as building blocks in the chemical synthesis of pharmaceutical compounds [94]. In further work on L-cysteine export with *E. coli*, 33 putative drug transporter genes were assayed, and of these overexpression of *acrD*, *acrEF*, *bcr*, *cusA*, *emrAB*, *emrKY*, *ybjYZ*, and *yojIH* was found to partially reverse growth inhibition by added L-cysteine [30]. As expected, also the intracellular L-cysteine concentrations were reduced upon expression of these carriers. Of particular relevance was Bcr, which is known from prior work to confer resistance to bicyclomycin or sulfathiazole. Overexpression of *bcr* resulted in a roughly fivefold increase in L-cysteine accumulation as compared to the control [30]. Comparisons of YdeD and Bcr showed that in some of the systems studied, Bcr is more effective than YdeD.

Besides YdeD, YfiK, and Bcr, a fourth carrier contributes to L-cysteine export [33]. This is CydDC, which belongs to the ATP-binding cassette (ABC) family of transporters, but does not interact with a cognate periplasmic binding protein as is typical of ABC transporters exporting molecules from the cytoplasm [95]. Assays with inverted vesicles demonstrated that CydDC transports glutathione and, to a lesser extent, cysteine from the cytoplasm to the periplasmic space in an ATP-dependent manner [33, 96]. The exporter is necessary in *E. coli* for the assembly of cytochrome bd and periplasmic cytochromes and is important for maintenance of the optimum redox balance in the periplasm. In line with the export function, an increased resistance to cytotoxic levels of L-cysteine by strains that overexpress *cydDC* was observed [33].

Since L-cysteine readily oxidizes to cystine, also the influence of cystine on the physiology of *E. coli* may be of relevance for L-cysteine formation. As a putative carrier involved in cystine export, YijE has been identified [34], which belongs to the drug/metabolite transporter (DMT) superfamily of carriers (Table 1). The *yijE* gene is upregulated upon cystine addition. Its overexpression suppressed the slow growth of *E. coli* in the presence of high concentrations of cystine, whereas the inactivation of *yijE* increased sensitivity to cystine.

11 Involvement of Outer Membrane Proteins in Export

E. coli has inner and outer membranes with an intervening periplasmic space. Whereas the four transporters, YdeD, YfiK, CydDC, and Bcr, export L-cysteine from the cytoplasm via the inner membrane into the periplasmic space, tripartite carriers allow the export of molecules to the extracellular environment (Fig. 1). These tripartite pumps include an inner membrane transporter, an outer membrane protein, and a periplasmic adapter protein. The inner membrane transporter binds and translocates the export substrate. In cooperation with the periplasmic adaptor protein, the transporter recruits an outer membrane protein to form an exit duct, thus spanning the entire bacterial cell envelope [8]. TolC is such an outer membrane protein of *E. coli*, and a mutant with *tolC* inactivated showed hypersensitivity to L-cysteine [35]. Overexpression of *tolC* in a cellular background with enhanced biosynthesis led to reduced degradation of L-cysteine, and increased extracellular accumulation to 1.13 g L^{-1} as compared to 0.96 g L^{-1} for the control, thus demonstrating the relevance to expelling L-cysteine from the cell. However, the inner membrane transporter and the periplasmic adapter protein involved in TolC-dependent L-cysteine export are unknown. TolC of *E. coli* can work with different export carriers including ATP-binding cassette transporters (e.g., MacB), resistance-nodulation-division transporters (e.g., AcrB), and transporters belonging to the major facilitator superfamily (e.g., EmrB). However, individual deletion mutants for *macB*, *acrB*, and *emrB*, as well as that of *macA*, *acrA*, and *emrA* encoding the respective periplasmic adaptor proteins, did not exhibit increased L-cysteine sensitivity [35], thus supporting the idea that either still unknown

transporters and periplasmic adapter proteins are involved in reducing the high cytosolic L-cysteine by direct export via the cell envelope or that TolC helps to reduce a high periplasmic L-cysteine concentration by diffusible transport. Other examples where TolC is involved in small molecule synthesis are the production of free fatty acids [97] and of geraniol with *E. coli* [98]. In both cases the tripartite carrier AcrAB–TolC is responsible for most of the efflux.

Also the outer membrane represents a diffusion barrier, and porins enable the flux of metabolites over this membrane [58]. The trimeric proteins form water-filled open channels that allow the passive penetration of hydrophilic molecules. *E. coli* produces more than ten outer membrane proteins, and it is not surprising that such proteins were also studied with respect to their influence in overcoming the outer membrane as the diffusion barrier for production purposes. Thus, there is an indication that overexpression of *ompW* influences L-threonine production [78], and *ompW* was also demonstrated to participate in small drug resistance [99]. Phenylpropanoids such as resveratrol are of interest as food additives and in health care products. Limited natural sources have promoted studies on their synthesis with *E. coli* [100]. Using proteomic studies as well as overexpression and gene silencing, the outer membrane proteins OmpA and FadL are likely to play a role in the transmembrane export of phenylpropanoids in *E. coli* [101].

12 Export of Branched-Chain Amino Acids and L-Methionine

In *C. glutamicum*, the export of branched-chain amino acids (BCAAs) and L-methionine is mediated by BrnFE [36, 37]. The exporter is a two-component permease where both transmembrane proteins act together as the carrier. A mutant deleted of both genes exhibits reduced L-isoleucine excretion, whereas an overexpressing strain exports this amino acid at an increased rate. As judged from internal amino acid concentrations and excretion rates, the exporter uses L-isoleucine, L-leucine, and also L-methionine at comparable rates, whereas the rate of L-valine is reduced. The expression of the *brnFE* operon is transcriptionally activated by the leucine-responsive protein Lrp, whose expression is controlled by autoregulation [36, 37, 102]. Interestingly, in an engineered L-leucine producer, the expression of the *brnFE* genes is upregulated, apparently as a consequence of the increased cytosolic L-leucine concentration [103]. Native control of *brnFE* expression and of BrnFE activity is apparently sufficient for effective L-leucine formation since *brnFE* overexpression in a high-level producer did not further increase its accumulation. As already mentioned, also L-methionine is exported by BrnFE, and under conditions where cytoplasmic L-methionine does not exceed a concentration of 50 mM, BrnFE is the dominant export system for this amino acid [37]. Also L-methionine acts as a coinducer of Lrp-dependent *brnFE* expression. Indeed, L-methionine is a better coinducer than L-isoleucine, indicating that methionine rather

than isoleucine might be the native substrate of BrnFE. In classically obtained L-isoleucine producers of *C. glutamicum*, the consequences of engineered L-isoleucine transport were studied. A strain with deleted uptake carrier *brnQ* and overexpressed *brnFE* accumulated 221 mM L-isoleucine [104], and a strain with *lrp* and *brnFE* overexpressed accumulated 205 mM L-isoleucine [105].

In *E. coli*, YgaZH is homologous to BrnFE. The minimal inhibitory concentration (MIC) of the L-valine analogue D,L-norvaline is dependent on the presence and copy number of *ygaZH* [106], as is the MIC of the L-isoleucine analogue D,L-4-thiaisoleucine. In a model strain, the final L-isoleucine concentration obtained was 1.11 g/L as compared to 0.32 g/L obtained without *ygaZH* amplification, and the strain engineered to produce L-isoleucine and expressing *ygaZH* was able to produce 9.46 g/L of L-isoleucine [38]. YgaZH is also beneficial for L-valine formation. The accumulation of a strain forming 2.38 g/L L-valine was almost doubled to 5.25 g/L due to *ygaZH* overexpression [106].

There is strong evidence that the carrier YeaS (LeuE) of *E. coli*, demonstrated to use L-threonine [19], is also involved in L-leucine excretion [24]. In expression control of *yeaS*, the global regulator Lrp is involved together with L-leucine, α-aminobutyric acid, and several other amino acids acting as coinducers. The BCAAs are not only of interest as a feed additive but also for human health. In this regard, the content of BCAAs in vinegar is of concern. Vinegar made by *Gluconacetobacter europaeus* contains 0.13 mM L-valine and no L-leucine [39]. In an effort to increase the BCAA content, *lrp* was deleted in *G. europaeus*. Comparative transcriptome analysis verified increased expression of BCAA biosynthesis genes and also that of *leuE*. This approach resulted in 0.48 mM L-valine and 0.11 mM leucine accumulation and is an indication that the exporter LeuE is involved in the export process of BCAAs in this Alphaproteobacterium.

13 Alanine Export by *E. coli*

Inducible L-alanine export was demonstrated using an *E. coli* strain unable to degrade L-alanine and using Ala-Ala dipeptide supply [60]. A mutant with increased Ala-Ala sensitivity enabled the isolation of four complementing chromosomal fragments [40]. Two uncharacterized carrier genes, *ygaW* and *ytfF*, and two characterized genes, *yddG* and *yeaS*, were identified. YeaS is able to accept small molecules such as L-threonine or L-leucine, and YddG is known to accept various aromatic compounds (Table 1). Individual overexpression of each of the four genes resulted in a decrease of the intracellular L-alanine level and an increase of the L-alanine export rate, demonstrating that the four carriers accept L-alanine at the high cytosolic concentration of up to 120 mM prevailing in the dipeptide assay. Among the four genes identified, YgaW exhibited the most striking impact on both the intra- and the extracellular L-alanine concentrations. Expression of the gene is induced at elevated concentrations of L-alanine, and even at low concentrations of this amino acid, the overexpression of *ygaW* causes L-alanine excretion. The gene

was therefore named *alaE*. Its use in a model strain caused a 22.5% increase in L-alanine accumulation without influencing growth or glucose consumption.

14 Glutamate Export by *C. glutamicum* and *E. coli*

L-Glutamate is traditionally produced by *C. glutamicum*. The excretion of this product was associated from the very beginning with membrane permeability [72]. Pioneering studies using mutants with inactivated oxoglutarate dehydrogenase activity and exhibiting constant L-glutamate efflux eventually led to the identification of the mechanosensitive channel encoded by NCgl1221 which is necessary for L-glutamate efflux [41]. Details on L-glutamate production depending on this channel are described in Chapter "Glutamate Fermentation Mechanism of Overproduction in Corynebacterium" of this book. Also the interplay of the unique pyruvate–oxoglutarate dehydrogenase supercomplex and the small autoinhibitory protein OdhI influences L-glutamate excretion [107]. Interestingly, the enterobacterium *Pantoea ananatis* is able to grow at acidic pH and is resistant to saturating concentrations of L-glutamic acid, both traits being advantageous for large-scale production. *P. ananatis* has been engineered to excrete L-glutamate in high concentrations dependent on the native carrier YhfK, and this is described in detail in Chapter "New Functions and Possible Applications of Amino Acids" of this book.

15 Diaminopentane Export by *C. glutamicum*

1,5-Diaminopentane is of interest as a component required for the synthesis of polyamides made from a diamine plus a dicarboxylic acid. The best known member of this class of polyamides is nylon, which is made from hexamethylene diamine plus adipic acid. Currently, about 6,000,000 tonnes of nylon and its related polyamides are produced annually. All this is derived from oil-based material, and the easy-to-produce diaminopentane with microbes opens up the opportunity of also producing the specific polyamide-containing diaminopentane by a bio-based process [108]. Since L-lysine production by *C. glutamicum* is well established, and L-lysine can be decarboxylated simply by expression of heterologous L-lysine decarboxylase, strains were engineered to generate 1,5-diaminopentane [43]. Up to 88 g L^{-1} of diaminopentane accumulation is reported [109]. The L-lysine exporter LysE is not involved in the export of diaminopentane. Instead, export is catalyzed via a carrier encoded by cg2893 identified by genome-wide transcription analysis and which was upregulated in a diaminopentane producer. The permease belongs to the major facilitator superfamily of transporters with great similarity to small metabolite efflux pumps. Its deletion resulted in a 90% reduced diaminopentane accumulation, and its overexpression revealed a 20% increased yield [43]. The carrier gene is apparently controlled by the adjacent gene cg2894 encoding a transcriptional regulator of the TetR type whose deletion also caused increased

diaminopentane accumulation. Further candidate carriers contributing to diaminopentane efflux are encoded by cg2181, cg2184, and cg2941 [43]. In *E. coli*, the carrier CadB is part of the lysine-dependent acid resistance system. It functions as a lysine/cadaverine antiporter, importing L-lysine and exporting diaminopentane. In *C. glutamicum*, expression of *cadB* together with L-lysine decarboxylase improved diaminopentane formation at the expense of L-lysine accumulation [110]. Not unexpectedly, the cg2893 encoded exporter also increases 1,4-diaminobutane accumulation by 24% up to a final concentration of 4 mM [111].

16 Export of Dipeptides

L-Glutamine is a nutritionally important amino acid for humans, but has low solubility and is unstable in solution. This can be solved by the use of the dipeptide L-alanyl-L-glutamine (Ala-Gln), which is a highly soluble and stable glutamine source [112]. Expression of an L-amino acid α-ligase of *Bacillus subtilis* in *E. coli* together with additional engineering to increase L-alanine and L-glutamine supply, as well as inactivation of a dipeptide-degrading activity, yielded a strain producing more than 100 mM Ala-Gln in a 5-L jar fed-batch cultivation [113]. However, growth of the peptide-producing strains was inhibited, and inhibition was also observed by addition of some dipeptides, including Ala-Gln, which was due to the peptides themselves and not their component amino acids. To identify the expected carrier, 34 putative exporter genes were overexpressed [29]. Of these, *bcr*, *norE*, *ydeE*, and *yeeO* conferred resistance to Ala-Gln or Gly-Tyr. The individual overexpression of each of these genes caused a 1.4- to 3.0-fold increase in Ala-Gln production, with *ydeE* and *bcr* being most effective. The four carriers isolated also utilize Ala-Val, Ala-Leu, and Ala-Ile as substrates. In addition to dipeptide export, YeeO also catalyzes the export of flavins [114], and Bcr also that of L-cysteine [30], sulfathiazole [115], and the peptide antibiotic bicyclomycin [116].

17 Export of Succinate

The diester of succinic acid, diethyl succinate, is of interest as an environmentally friendly solvent and as a raw material for biodegradable plastics [117]. Potent production strains are anaerobes such as *Anaerobiospirillum succiniciproducens* and *Mannheimia succiniciproducens*, as well as engineered *E. coli* strains. Also *C. glutamicum* can form succinate in the absence of oxygen from sugars via the reductive TCA cycle [118]. Two different approaches, one using a comparative transcriptome analysis [44] and the other a comparative genome analysis [9], resulted in the export carrier SucE of *C. glutamicum*, which participates in succinate efflux. The gene is threefold upregulated under microaerobic conditions [44], and

its deletion reduces in part succinate accumulation [9]. In a strain with deleted lactate dehydrogenase, overexpression of *sucE* increases the succinate concentration from 174 mM to 274 mM [44]. SucE belongs to the aspartate:alanine exchanger (AAEx) family transporters (Table 1). With an engineered *C. glutamicum* strain, 1.13 M of succinate with a yield of 1.67 mol mol (glucose)$^{-1}$ is obtained [119]. However, this was achieved without *sucE* overexpression, suggesting that the native regulation of this gene and SucE activity is sufficient for high extracellular accumulation of succinate or indicating that further carriers are involved.

18 Export of Nucleosides

Sodium salts of inosine monophosphate (IMP) and guanosine monophosphate (GMP) are potent flavor enhancers. They are widely used as food additives in combination with monosodium glutamate to synergistically increase umami flavor. They are made industrially starting from the corresponding nucleosides produced by engineered microorganisms such as *Bacillus amyloliquefaciens*, *Bacillus subtilis*, and *E. coli* [120]. Two carrier genes are known so far to be of relevance for production purposes. One is *pbuE* (*ydhL*) present in *B. subtilis* and *B. amyloliquefaciens* which encodes a purine base and purine nucleoside efflux pump [121]. Its expression is controlled by an adenine-dependent riboswitch with the control mechanism also retained when expressed in *E. coli*. Overexpression of *pbuE* enhanced inosine production by a *B. amyloliquefaciens* nucleoside-producing strain. *pbuE* overexpression was also found to increase 5-aminoimidazole-4-carboxamide (AICA) ribonucleoside accumulation, indicating that the substrate specificity of the PbuE pump extends to this nucleoside [45]. Whereas PbuE accepts both purine base and purine nucleosides as substrate, the second exporter, YicM (Nepl) of *E. coli*, accepts purine nucleosides only and not the free bases [46]. It was found that selection for resistance to 6-mercaptopurine and its overexpression led to an enhanced rate of inosine excretion by an inosine-producing *E. coli* strain. Also heterologous overexpression of the *nepl* gene led to increased product formation in a *B. amyloliquefaciens* strain, which again confirms the functionality of *Nepl* as a nucleoside efflux permease [45]. In *C. glutamicum* an efflux permease with structural similarity to *nepl* is present and is designated *cepA* [47]. Its overexpression increased resistance of *C. glutamicum* to the purine analogues 6-mercaptopurine and 6-mercaptoguanine. Interestingly, this strain also showed a slightly increased resistance to the antibiotics nalidixic acid and ampicillin, which once again raises the question of the natural function of such an exporter.

19 Export of Biofuels

Whereas to date export has largely been described from the aspect of increased external accumulations, another aspect is preventing the internal accumulation of the biosynthesis product in order to counteract cytotoxic effects. Overcoming the toxicity of biofuels poses a great challenge. As convincingly demonstrated in the past few years, such fuels can also be synthesized by microorganisms [122]. Fuels are inherently toxic due to their solvent-like properties, but bacteria such as *Pseudomonas putida* are able to grow in the presence of 90% (v/v) toluene and other solvents due to solvent efflux pumps being present [123]. The efflux carriers of *P. putida* include the tripartite carriers TtgABC, TtgDEF, and TtgGHI (Fig. 1). Using a bioinformatic approach, a set of 43 potential tripartite efflux pumps was selected, and genes were overexpressed in *E. coli* and assayed for survival in representative biofuels [48]. In addition to AcrAB from *E. coli*, a carrier from *Alcanivorax borkumensis* resulted in increased resistance to limonene which is of interest as a precursor for jet fuel. *E. coli* strains overexpressing the carrier of *A. borkumensis* improved limonene accumulation significantly [48]. In addition, using directed evolution, AcrB muteins were isolated, which improved the excretion rate for *n*-octane 1.5-fold and for α-pinene 4.0-fold [124]. Isopentenol is another interesting biofuel. The carrier gene *mdlB* was identified in the search for genes of *E. coli* overexpressed in the presence of this alcohol [53]. MdlB is the ATP-binding component of the ABC carrier involved in lipid A export. Its overexpression resulted in a 12% improvement in isopentenol production. ABC transporters were also assayed for improvement of isoprenoid-producing *E. coli* in a system where cultures were grown with an overlay of decane. A panel of 16 ABC carriers was assayed for increased secretion or partitioning into the biocompatible decane phase [54]. With MsbA of *E. coli*, a 4.4-fold canthaxanthin accumulation was achieved; and with MsbA of *Salmonella enterica* ser. *typhimurium*, a 2.4-fold zeaxanthin accumulation was achieved.

Also in *Saccharomyces cerevisiae*, ABC exporters are identified as being related to fuel tolerance. The overexpression of the two efflux pumps Snq2p and Pdr5p increases the tolerance of the yeast for decane and undecane by reducing the intracellular levels of these alkanes [55]. This is similarly the case with the efflux pumps ABC2 and ABC3 of *Yarrowia lipolytica* when expressed in *S. cerevisiae* [56]. Increased expression of another ABC export carrier present in *S. cerevisiae*, ADP1, prevented growth inhibition by adding up to 7.5% (v/v) ethanol, and the ethanol productivity was approx. 20% higher in the presence of ethanol [57].

20 Outlook

Microorganisms can be used to produce a wide range of small molecules such as antibiotics, amino acids, and fuels. In all these cases, it is essential to remove the molecule from the cytosol and transport it outside the cell in order to maintain low concentrations of this product which is toxic for the cell and to obtain high extracellular product concentrations. The proteins involved in transport can naturally serve to export the molecule and can be directly assigned to the molecule by means of genome information. This is, for example, often the case with antibiotics, where the synthesis genes in the operon may be present together with the corresponding transport gene [125], and can thus influence product formation [98, 126]. The situation is different with biofuels. In this case, it is first necessary to establish a metabolic pathway, frequently by using heterologous genes, and the specific exporters are generally unknown.

The significance of exporters for the formation of amino acids has been convincingly demonstrated. The exporters are frequently unspecific, and most of them are probably not designed as specific amino acid exporters. This could also be the case for LysE from *C. glutamicum*, which may originally have functioned as a canavanine exporter and may have been acquired by gene transfer. Since no conclusions can be drawn about the specificity of an exporter for a particular amino acid on the basis of the genome organization, further efforts are necessary to identify carriers, their specificity, and to verify their significance for amino acid export.

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Novel Technologies for Optimal Strain Breeding

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Abstract The implementation of a knowledge-based bioeconomy requires the rapid development of highly efficient microbial production strains that are able to convert renewable carbon sources to value-added products, such as bulk and fine chemicals, pharmaceuticals, or proteins at industrial scale. Starting from classical strain breeding by random mutagenesis and screening in the 1950s via rational design by metabolic engineering initiated in the 1970s, a range of powerful new technologies have been developed in the past two decades that can revolutionize future strain engineering. In particular, next-generation sequencing technologies combined with new methods of genome engineering and high-throughput screening based on genetically encoded biosensors have allowed for new concepts. In this chapter, selected new technologies relevant for breeding microbial production strains with a special emphasis on amino acid producers will be summarized.

Keywords Conjugation, CRISPR-Cas9, FACS, Genetically encoded biosensors, Metabolism, Recombineering, Regulation, Transcriptional regulators

Contents

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1 Introduction

The rapid development and improvement of microbial production strains capable of converting renewable carbon sources into value-added products is an important aspect in the establishment of a knowledge-based bioeconomy [1]. In the field of amino acid fermentation, efficient producer strains for a number of amino acids have already been developed by classical strain breeding and/or rational engineering. Nevertheless, there is still room for improvement of these strains and there are some amino acids for which efficient producer strains are not yet available. Furthermore, our molecular understanding of overproduction is still unsatisfactory in many aspects.

The breeding of optimal producer strains is a complex process, involving random mutagenesis and screening, rational engineering, or reconstruction by introduction of a defined set of "productive" mutations. Despite the huge progress of metabolic engineering in the past decades, random approaches of strain development are still sometimes superior to rational approaches. This is known, e.g., for L-lysine [2] and L-threonine producers [3] but also for other small molecule producers, e.g., for artemisinin [4]. For L-threonine production with *Escherichia coli*, it is known that inactivation of 56 genes led to increased production although they are not involved in L-threonine synthesis [3]. Furthermore, genetic traits of overproducers are known without an understanding of their molecular basis. For example, in the L-lysine producer strain *Corynebacterium glutamicum* B-6 that was obtained by random mutagenesis, the genes of the L-lysine, L-arginine, and L-histidine biosynthesis pathways are overexpressed at the same time [5], and in the L-arginine producer *C. glutamicum* strain A-27, the cluster of arginine biosynthesis genes is extremely upregulated to a level not achievable by plasmid-based overexpression [6].

Against that background, a number of areas can be defined for which new technologies are desirable:

- Fast transfer of known mutations or DNA fragments into a desired genome in order to improve classically derived strains or construct novel producers from scratch, i.e., starting with a wild-type strain
- Rapid analysis of strains whose properties are not yet (completely) understood by omics methods (genomics, transcriptomics, proteomics, metabolomics) in order to decode favorable genomic alterations derived from random approaches
- Generation of new strains and of novel mutations by directed or random mutagenesis in order to continuously improve strains

In the era of systems and synthetic biology, novel tools have been established which allow to address the areas mentioned above and thus can contribute to an acceleration of strain development and to the generation of new knowledge for understanding the fundamentals and details of microbial metabolite overproduction. In this article, a number of new methods and technologies will be described that can be exploited for breeding producer strains for amino acids but also other small molecules. In addition, tools will be mentioned that would be desirable for strain development but are not yet available.

2 Recombineering and Its Advantages

Recombineering is a versatile and proven method for rapid transfer of mutations generating either insertions, deletions, or point mutations on chromosomal or episomal targets. It is a technique that replaces the tedious standard recombinant DNA techniques such as cutting DNA with restriction endonucleases, purifying DNA fragments, ligating DNA fragments, and transferring DNA into competent cells. It does not require any known recombination function of the host but utilizes bacteriophage recombination proteins to perform homologous recombination [7–9]. Recombineering is based on the phage λ recombination proteins Exo (Redα) and Beta (Redβ) or, alternatively, on the homologous proteins RecE and RecT of the prophage Rac [10–12]. Exo and RecE are double-stranded DNA (dsDNA)-dependent 5′-3′ exonucleases leaving 3′ssDNA overhangs. Beta and RecT are single-stranded DNA (ssDNA)-annealing proteins. Either ssDNA or dsDNA can be used as substrates for recombineering. According to the current model of the mechanism of λ Red recombination (Fig. 1), Beta binds to ssDNA and catalyzes its annealing to the lagging strand of the replication fork, where it is incorporated into the newly synthesized strand as part of an Okazaki fragment [13, 14]. Recombineering is used in *Escherichia coli* and other bacteria of biotechnological relevance, such as *C. glutamicum* [15], *Bacillus subtilis* [16], *Pantoea ananatis* [17], or *Lactococcus lactis* [18]. It is done with the phage λ or prophage Rac proteins mentioned above or homologous proteins which show favorable functionality in selected bacteria [15, 19, 20].

Since recombineering requires only ssDNA and the phage ssDNA-annealing protein (Beta/RecT) [21], synthetic oligonucleotides are most widely used, which carry in the middle the desired mutation and sequences on either side that are homologous to the chromosomal target region. With 90 bp oligonucleotides, mismatch mutations or exogenous sequences, each up to 30 bp, were introduced into the *E. coli* chromosome, with the efficiency correlating to the length of homologous sequence between the oligonucleotide and its chromosomal target [22]. Similarly, up to 45 kb of chromosomal sequence could be deleted with the efficiency of producing a deletion correlating to the size of the deletion. These results were obtained with special *E. coli* strains having defects in the methyl-

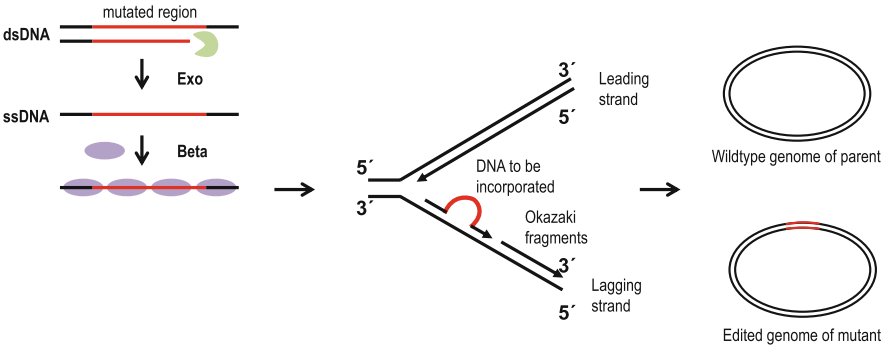

Fig. 1 The current model for recombineering. Editing DNA fragments may be either double-stranded (dsDNA) or single-stranded (ssDNA). If the editing DNA is double stranded, then one strand is digested by the λ Red Exo exonuclease. The Beta protein from λ Red binds the ssDNA and protects it while being escorted to a replication fork of the chromosome. The editing ssDNA anneals to the lagging strand, acting as an Okazaki fragment. The genomic location for integration is determined by complementary sequences flanking the mutation site. After DNA replication and chromosome segregation, some cells eventually carry the desired mutation

directed mismatch repair system [9] and easily recognizable phenotypes as possible with the LacZ or GalK system [23].

For a sizeable insertion, such as a new gene, a linear dsDNA substrate is necessary as well as the function of both the ssDNA-annealing protein (Beta/RecT) and the 5′-3′ exonuclease (Exo/RecE). The dsDNA can be generated as PCR product that contains 40–50 bp of flanking DNA at each end homologous to the chromosomal insertion site. These homologous regions are provided at the 5′-end of each synthetic primer. In this manner a successful and reliable integration of fragments up to 3.5 kb could be achieved [24, 25]. However, for large fragments, it becomes increasingly difficult to generate PCR product in sufficient quantity, and the increased size of these fragments makes transformation and integration significantly less efficient. To overcome this problem, "gene gorging" has been developed, where dsDNA fragments are generated in vivo by digestion of a donor plasmid with a nuclease (I-SceI) that does not cleave the host genome [26]. This allows the transfer of large fragments into the chromosome and yields recombinants with sufficient high frequency (1–15%) of even not easily accessible *E. coli* strains, when coupled to suitable selection strategies [27]. Such methods can be of interest for larger genome alterations of producer strains. When dsDNA recombineering is used to transfer fragments from the chromosome to a plasmid, the fragment size isolated can be up to 50 kb in length [28]. Recombineering with dsDNA to introduce larger fragments into targets has been established, for instance, for *E. coli* and *Pantoea ananatis* [17].

A major issue is of course the frequency of recombination. In general, ssDNA recombineering is more efficient than dsDNA recombineering. Efficiencies are in the range of 0.01% and can approach 1% [29, 30]. They vary largely in dependence of the host and the experimental conditions. If host mismatch repair is inactivated or

reduced, either by mutation or by using an oligonucleotide that creates a C–C mismatch, even a 20% recombination frequency is achievable [31]. Therefore, although simply performed in a single step, recombineering usually requires a screening or selection step. Useful methods for screening involve PCR amplifications, such as the mismatch amplification mutation assay-PCR (MAMA-PCR). MAMA-PCR is capable of identifying single base changes by screening colonies. It is also helpful if the mutation to be inserted creates a restriction site change that can be monitored by a diagnostic cut of the PCR-amplified fragment. As these methods still require a sufficiently high frequency of recombination, counterselection has been developed to avoid screening or to restrict screening to a confirmatory analysis. In this two-step procedure, first, a *cat-sacB* cassette (or another counter-selectable cassette) is placed on the DNA, and successful recombinants are retrieved as chloramphenicol resistant [7]. The cassette is then replaced with the desired alteration in a second recombineering event, and successful recombinants are identified by their sucrose resistance caused by the loss of *sacB*. The final construct will carry the desired alteration and have no drug marker. Other counter-selectable markers than *sacB* are available [12]. A recently presented technology uses the incorporation of an added nonnatural nucleoside into DNA due to *Herpes simplex* virus thymidine kinase (hsvTK) activity [32], with the consequent death of non-recombinants which have not lost hsvTK. The elegant procedure developed uses liquid handling only, without the need for a manual procedure for identifying the right recombinants by colony picking and conducting multiple PCR reactions for each colony. The virtually complete absence of false-positives in this procedure made it possible to introduce mutations at four different loci in subsequent rounds of recombineering without any need for colony isolation or genotype confirmation. Because the entire process is conducted via liquid handling only, it is adaptable for full automation, and different recombineering projects can be conducted in parallel in multi-well formats.

Due to its simplicity and speed, recombineering is an established tool to develop *E. coli* producers by introducing single mutations. In a further development, multiplex automated genome engineering (MAGE) was introduced as an evolutionary strategy for strain development, which targets many locations on the chromosome for modification across a population of cells [22]. MAGE takes advantage of the ultrahigh efficiency of ssDNA recombineering with *mutS* strains to achieve simultaneous editing of multiple targets in a selection-free format. It was applied to tune the ribosome-binding site of 24 genes documented to increase lycopene yield. A pool of 4.7×10^5 90-mer oligos was used containing degenerate ribosome-binding sites to simultaneously maximize lycopene production. Screening of variants that produced intense red pigmented colonies yielded clones which produced up to 1 mg lycopene per g cells and which carried up to five mutations. MAGE was also used for the simultaneous incorporation of T7 promoters into 12 genes associated with the synthesis of aromatic amino acids [33]. Up to 9 mg product per gram cells quantified as tryptophan-derived indigo was achieved. Although the product yields for both lycopene and tryptophan are far from being of industrial relevance, site-directed genomic diversity as feasible by

recombineering and MAGE might be useful to obtain variants and to optimize metabolic flux through biosynthesis pathways.

Among the variety of Gram-negative bacteria for which recombineering has been established, *Pantoea ananatis* is of particular interest since it has specific traits useful for small molecule production, such as growth at acidic pH and resistance to saturating concentrations of glutamic acid [34]. It has been observed that expression of the λ Red genes is highly toxic for *P. ananatis* wild type. However, a screening yielded a mutant resistant to the toxic effects of λ Red. Using this strain, procedures for fast introduction of multiple rearrangements to the *P. ananatis* genome based on the λ Red-dependent integration of ssDNA or short dsDNA fragments have been demonstrated [17].

Recombineering has also been established and applied for *C. glutamicum* [15, 35]. To assay for ssDNA recombineering in this bacterium, λ Beta and RecT as well as homologous proteins of *Corynebacterium aurimucosum* and of the mycobacteriophages Che9c and Halo were used (Table 1). The latter two proteins were chosen since both *Mycobacterium* and *Corynebacterium* belong to the order *Corynebacteriales*, and genes of *M. tuberculosis* show functionality in *C. glutamicum* [36]. With the exception of λ Beta, all proteins exhibited functionality. Kanamycin sensitivity due to a frameshift mutation in a chromosomally integrated kanamycin resistance gene was cured by the use of 1 μg of a "healing" 50-mer oligonucleotide in electroporation assays. The highest recombineering activity was obtained with RecT of the *E. coli* Rac prophage. Under optimized conditions, RecT enabled recombination frequencies in the wild type of *C. glutamicum* exceeding 10^6 recombinants per assay, which contained about 10^9 viable cells after electroporation. This is only about one order of magnitude away from that obtained with Beta in *E. coli* strains specifically engineered for high

Table 1 Comparison of recombinase efficiencies in a *C. glutamicum* test strain carrying the chromosomally integrated kanamycin resistance gene with an inactivating point mutation

Plasmid	Kanamycin-resistant cells per 10^9 viable cells		
	+ Oligonucleotide[a]	− Oligonucleotide	pJC1 control plasmid with kanamycin resistance
pCLTON2-bet	8	0	2.2×10^5
pCLTON2-recT	1.3×10^4	31	5.0×10^5
pCLTON2-gp43	9.7×10^1	57	4.3×10^5
pCLTON2-gp61	3.1×10^2	1	3.4×10^5
pCLTON2-rCau	2.5×10^3	7	2.9×10^5

Adapted from Binder et al. [15]

[a]1 μg of a 50-mer oligonucleotide repairing the inactivating point mutation in the kanamycin resistance gene was used. The indicated values are averages from at least three biological replicates

mutation frequency like a decreased activity of the methyl-directed mismatch repair system [9, 22]. This example illustrates the broad applicability of Beta/RecT homologs in bacteria for the efficient introduction of genomic mutations making it a versatile tool for rapid strain development.

In summary, recombineering is the method of choice for the rapid transfer of mutations and the delivery of new strains by aiding the creation of point mutations, deletions, or small insertions in the chromosome. The technique is most advanced and also commercialized for its application in *E. coli* [7] but emerging for various bacteria of biotechnological relevance [15, 17, 37]. The ideal system is plasmid based, does not cause background mutations, and works without screening.

3 The CRISPR-Cas System and Its Perspectives

Key component of the CRISPR-Cas system is an RNA-guided nuclease introducing double-strand breaks (DSBs) into DNA [38]. The system is extremely attractive for the engineering of DNA at any given site and established for a plethora of organisms including yeast, zebra fish, mosquito, or plants and already used to correct monogenic disorders in mouse and human cell lines [39, 40]. In eukaryotes, it is the leading genome-editing tool which facilitates engineering of a cell's genome at a level of efficiency and/or precision previously unattainable with other systems, such as zinc finger nucleases [41, 42] or transcription activator-like (TAL) effector nucleases [43–45]. The systems in use are built on components from clustered, regularly interspaced, short palindromic repeat (CRISPR)-associated Cas (CRISPR-associated) systems evolved in Bacteria and Archaea as immune systems against foreign nucleic acids. The most commonly used RNA-guided nuclease is based on the type II CRISPR-Cas9 system from *Streptococcus pyogenes* [39]. In the native system Cas9 is guided to its target sequence by an RNA hybrid derived from two processed RNAs, crRNA (CRISPR RNA) and tracrRNA (trans-activating crRNA). However, a single chimeric small-guide RNA (sgRNA) was constructed combining tracrRNA and crRNA features and addressing the nuclease Cas9 as a ribonucleoprotein complex to the target site [46]. The target site consists of a nucleotide stretch matching the 5′-terminal sgRNA sequence (usually 20 bp in length) followed by a short nucleotide sequence called protospacer adjacent motif (PAM), which is NGG in the case of *S. pyogenes* Cas9 (Fig. 2). The fact that target sequence specificity of the RNA-guided nuclease is governed by RNA–DNA hybridization confers the versatility to the system. Only the nuclease and an sgRNA is required with 20 bp nucleotide sequence complementary to the target site with three nucleotides NGG adjacent (the PAM). In addition to native Cas9 introducing a DSB at the target locus, engineered Cas9-derivatives are available, like Cas9n introducing a single-strand "nick," or dCas9, which has maintained its sequence-specific dsDNA-binding capability but which is catalytically inactive, and a fusion of dCas9 with the omega subunit of *E. coli* RNA polymerase suitable to activate gene expression [47]. The recent advances in

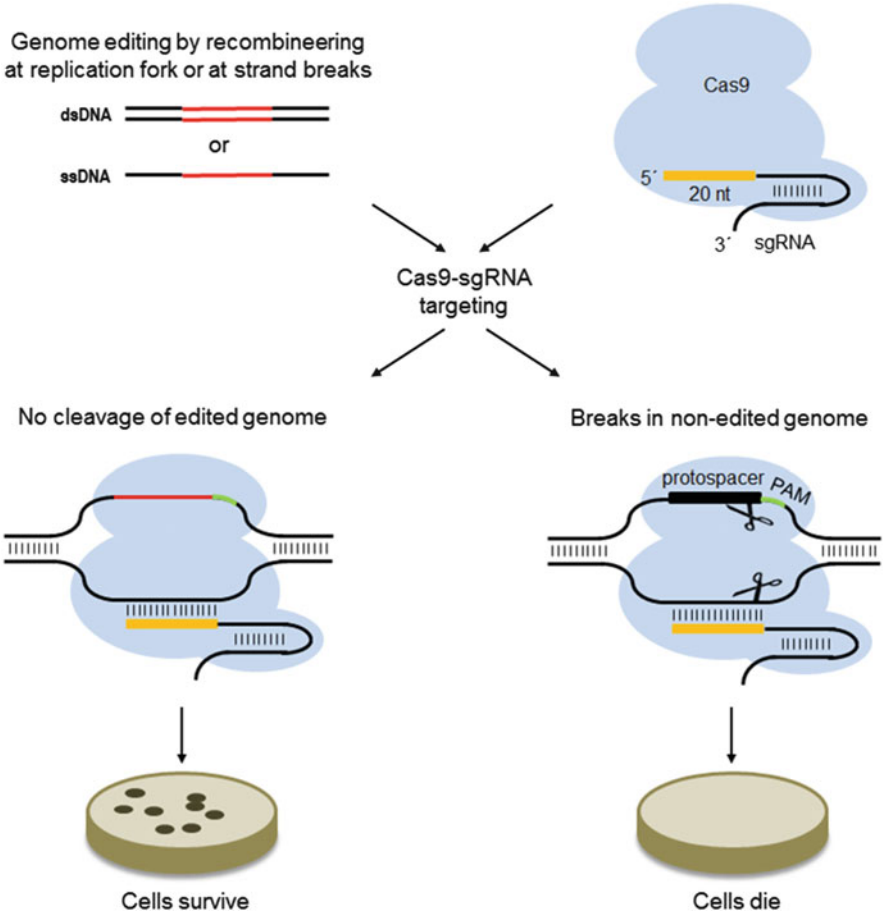

Fig. 2 Application of the CRISPR-Cas9 system for efficient genome editing. The template carrying the mutant allele is either double-stranded DNA (dsDNA) or single-stranded DNA (ssDNA). Their integration into the genome is assisted by recombineering using the λ Red system or by Cas9-cleavage activity. The endonuclease Cas9 requires a small-guide RNA (sgRNA) which is composed of a "scaffold" sequence necessary for Cas9 binding and a user-defined ~20 nucleotide "spacer" or "targeting" sequence which defines the genomic target to be modified. Juxtaposed to the DNA complementary region is the protospacer with the protospacer adjacent motif (PAM) immediately upstream. The PAM sequence is absolutely necessary for target binding and consists in the case of Cas9 of *Streptococcus pyogenes* of the short motif 5′-NGG-3′. With the sgRNA designed to target the "unedited" genome, the Cas9-sgRNA complex cuts unedited target DNA, resulting in a double-strand break, while edited DNA is immune to cleavage by the Cas9-sgRNA complex. As a consequence, the double-strand breaks result in death of cells, whereas edited cells survive

CRISPR-Cas technology herald beneficial opportunities in bacterial strain development. The current advantages demonstrated are (1) access to engineer recalcitrant strains, (2) use as an extremely efficient counterselection system, (3) use in gene regulation, (4) advantageous reduction in genome manipulation time, and (5) the possibility of multiplex genome editing and regulation.

The lethal DSB at the target locus effectively acts as a selection against wild-type sequences during genome editing and increases at the same time the rate of recombination at this locus when homologous DNA is supplied. This enables the use of CRISPR-Cas genome editing even without phage recombinases expressed and gives access to genome editing in bacteria that were previously recalcitrant to specific genome manipulation. In the landmark work by Jiang et al. [48], *Streptococcus pneumoniae* targets were engineered by co-transformation with both the targeting and editing templates, which led to a recovery of close to a 100% of edited cells. *Clostridium* species are of much biotechnological interest, but genetic tools are still limited. *Clostridium beijerinckii* is a prominent solvent producer, and the use of CRISPR-Cas technology enabled to introduce a 262 bp chromosomal deletion into the chromosome [49]. The plasmid used encoded the targeting CRISPR-Cas nucleoprotein components together with a genomic fragment containing the deletion. Its application resulted in clones all carrying the attempted chromosomal deletion.

In broader use is CRISPR-Cas genome editing in combination with recombineering where it is used as an efficient counterselection system (Fig. 2). It was used for *Lactobacillus reuteri* and ssDNA recombineering [50]. Introduction of up to five adjacent base changes into the chromosome were recovered at efficiencies of more than 90%. However, CRISPR-Cas enabled to introduce deletions at different loci of up to 702 bp with oligonucleotides and efficiencies of up to 10%. The general use of CRISPR-Cas has been demonstrated for *E. coli* and ssDNA recombineering by allelic exchange of a plasmid-encoded mutation [48]. The use of CRISPR-Cas and dsDNA recombineering increases the efficiency of genome editing significantly. To introduce an 804 bp deletion in *cadA* of an *E. coli* strain equipped with Cas9 and the λ recombination proteins, an appropriate PCR fragment was co-transferred with a plasmid encoding the targeting sgRNA [51]. The mutation efficiency obtained was 69%, enabling the easy recognition of the deletion by PCR analysis. When the same *cadA* deletion was done by delivering the mutated allele on a plasmid, the mutation efficiency was increased to 86%. This high efficiency is likely due to the fact that multiple copies of the allelic DNA are present in the cell increasing the number of recombination events, as known for "gene gorging" [26]. Thus, CRISPR-Cas allows dsDNA recombineering with a very high efficiency, which without CRISPR-Cas application is below 0.01% [52]. More importantly, it operates with this high efficiency also in *E. coli* wild-type strains not carrying mutations in the methyl-directed mismatch repair system to increase recombineering frequencies [53].

Since the native CRISPR-Cas system is designed to synthesize several targeting RNAs at the same time, it offers the opportunity to edit in one host cell several loci in a single experiment (multiplexing). For a single-step double deletion of *srtA* and

bgaA in *S. pneumoniae*, a targeting construct was used matching *srtA* and *bgaA* as present in the wild type [48]. Co-transformation with both editing templates at the same time showed that editing at the two different loci occurred in 6 out of 8 cases. In *E. coli* MG1655, a double deletion was introduced with a plasmid-encoded editing template at a frequency of 97% [51]. Even a triple deletion was possible in *E. coli* MG1655 at a frequency of 47%, and a mixed gene deletion plus a gene insertion occurred at a frequency of 78%. CRISPR-Cas has been used for single gene editing and multiplexing also in *Streptomyces*. *Streptomyces* species are well-studied producers of diverse secondary metabolites. Genomic deletions can be made via homologous recombination, which is labor and time intensive and requires several screening steps. Using a conjugative plasmid encoding Cas9 together with the targeting and editing templates, a 12 kb region could be deleted in *S. albus* in two of three exconjugants analyzed [54]. By multiplexing two different loci in *S. lividans*, 4 out of 8 exconjugants were correctly edited. In a similar approach, the genomic regions of actinorhodin and undecylprodigiosin synthesis in *S. coelicolor* were targeted by multiplexing. The corresponding simultaneous deletion of the 21.3 kb and 31.6 kb region occurred with an efficiency of 45% [55]. Notably, the total time required for one round of genome engineering in *Streptomyces* was decreased to at least one-half of that of conventional methods.

A catalytically dead Cas9 (dCas9) lacking endonuclease activity, when co-expressed with an sgRNA, generates a ribonucleoprotein complex that can specifically interfere with transcription factor binding, RNA polymerase binding, or transcriptional elongation and therefore used to control gene expression [46]. By directing dCas9 to the promoter region of *bgaA* in *Streptococcus pneumoniae*, an up to 14-fold reduction in β-galactosidase activity was obtained depending on the targeted position [47]. In *E. coli* a greater than 100-fold reduction in GFP fluorescence was observed by targeting of *gfp* promoter regions overlapping or adjacent to the −35 and −10 promoter elements and to the Shine–Dalgarno sequence [47]. Targets on both strands showed similar repression levels, suggesting that the binding of dCas9 to any position within the promoter region prevents transcription initiation. With the red fluorescent protein (mRFP)-based reporter system inserted into the *E. coli* genome, a 10- to 300-fold repression was demonstrated with sgRNAs targeting the non-template DNA strand of the mRFP-coding region [56] illustrating blocking of transcription [53]. The functionality of dCas9 to control gene expression has also been shown for *S. coelicolor* by reducing expression of *actIORF1*, thus preventing formation of the blue-pigmented polyketide antibiotic actinorhodin [57]. In *Corynebacterium glutamicum*, CRISPR interference with dCas9 was used to reduce expression of *pgi* (phosphoglucose isomerase) to increase L-lysine production and of *pck* (PEP carboxykinase) and *pyk* (pyruvate kinase) to enhance L-glutamate synthesis [58]. Interestingly, the CRISPR-Cas system was also used for activation of gene expression in *E. coli* [47]. To convert dCas9 into a transcriptional activator, it was fused to the ω-subunit of RNA polymerase and directed toward *gfp-mut2* under control of a weak synthetic promoter. The dCas9-ω fusion located 80 or 96 nucleotide upstream of the transcriptional start site provided a 7.2- and 23-fold

induction, respectively. This is a tool of interest to study consequences of increased gene expression on product formation.

In conclusion, CRISPR-Cas increases the efficiency of genome editing dramatically. Bacteria recalcitrant to specific genome manipulation can be modified with this technique, and in combination with recombineering, very high efficiencies are obtained. Further developments of this new and promising editing system are required to make it available for various bacteria of biotechnological interest and in particular to enable its comfortable use in consecutive rounds of strain development.

4 Think Big: Large Genome Alterations

Besides technologies for rapid site-specific introduction of small genome changes like point mutations or small deletions or insertions, the era of genomics and synthetic biology also has motivated studies aiming to explore the influence of large genome alterations on growth physiology and product formation. There are three major directions in this field: (1) bottom-up synthesis of designed genomes and transfer into a suitable host, (2) top-down reduction of the genome size to reduce complexity and discard unnecessary or disturbing genes, and (3) transfer and exchange of larger DNA fragments between genomes and episomes. These will be described below.

4.1 Maximal Output with Minimal Genome

The concept of the minimal cell has fascinated scientists for a long time. There is interest in minimal genomes from basic science to reduce or redesign genomes of selected microbial model species. This has culminated in the total synthesis of the 1.08 Mbp *Mycoplasma mycoides* JCVI-syn1.0 genome starting from digitized genome sequence information and its transplantation into a *Mycoplasma capricolum* recipient cell to create new *M. mycoides* cells that are controlled only by the synthetic chromosome [59]. This bottom-up approach of genome synthesis obviously represents the most straightforward procedure to design and implement producer cells from scratch. However, the difficulties in transferring large DNA fragments >500 Mbp into recipient cells have prevented the application of this approach in strain development hitherto. The top-down approach involves the reduction of genome size motivated by the assumption that a strain with a minimized genome harboring only the necessary functions for growth and product formation will strongly improve modeling, predictability, and engineering, allowing the construction of producer strains with superior properties [60].

In approaches to systematically reduce the genome size of *E. coli*, single deletions were introduced with the largest one exceeding 300 kb [61]. Suitable

techniques include the FLP/FRT, the Cre/loxP, or the λ Red system, all three of them using two recombinase recognition sites on the same DNA molecule [62]. Also the simultaneous deletion of a non-contiguous region of 54 kb using large synthetic DNA was achieved in this manner [63]. Serial combination of single deletions enabled to delete 38.9% of the chromosome of *E. coli* K-12 [61]. The deletions cause a number of unpredictable effects influencing growth, transformation efficiency, morphology, and other properties [64]. Frequently, the impact on fitness is negative, but it can also be positive. Single-gene deletions could activate cellular metabolism [65, 66], and the synergistic effect of multiple deletions could enhance transformation frequency [64] or improve production of proteins as shown for *B. subtilis* [67] and *C. glutamicum* [68]. *E. coli* DGF-298 with 35.2% of the genome deleted and a 2.98 Mbp genome showed a better cell yield in a rich medium than the wild-type K-12 strain and was not auxotrophic [69]. The downregulation of genes encoding chaperones and proteases has been discussed as one reason for the better cell yield of this strain. However, it is difficult to rationally find gene deletion combinations that produce synergistic effects supporting or activating cellular functions, especially production of a small molecule. Growth, productivity, and yield are the results of a functional network of hundreds of genes, and this network is still difficult to elucidate or to design in view of our incomplete knowledge of gene functions and regulatory effects.

In two independent studies, the genome reduction approach was assessed in relation to L-threonine formation with *E. coli* K-12 derivatives [70, 71]. In one of them growth of mutants carrying single and stepwise combined deletions was judged in M9 minimal medium to maintain robust cell growth [71]. The genome of the final strain MGF-01 was reduced by 1.03 Mbp (22% genome reduction), and it grew as well as the wild type in the exponential phase and continued growing after the wild type had entered the stationary phase. The final cell density of MGF-01 was 1.5 times greater than that of the original W3110 strain, which was attributed to reduced acetate formation. Removing feedback control of aspartate kinase, which controls flux toward L-threonine, by introducing the mutation *thrA345* and preventing flux toward L-methionine by deleting *metA* resulted in a strain that accumulated 10.6 g/L L-threonine, whereas the non-genome-reduced strain carrying the same two mutations produced 4.4 g/L L-threonine [71]. In another study strain MDS42 was constructed with the genome reduced by 0.66 Mb (14%) [64]. The focus of this latter study was to obtain a genetically stable strain by removing mobile DNA elements that mediate recombination events such as transposition and horizontal gene transfer, including insertion sequence (IS) elements, transposases, defective phages, integrases, and site-specific recombinases. Growth in minimal medium and protein production of strain MDS42 was similar to that of the wild-type MG1655, and as unanticipated beneficial properties, the electroporation efficiency was increased and recombinant genes and plasmids were more stable than in other strains. MDS42 also served as a basis to construct an L-threonine producer [72]. The mutations introduced included in this case the overexpression of a feedback-resistant threonine operon (*thrA*BC*) under the control of a recombinant *tac* promoter, the deletion of the

genes that encode threonine dehydrogenase (*tdh*) and threonine uptake proteins (*tdcC* and *sstT*), as well as the introduction of a mutant threonine exporter gene (*rhtA23*). The strain was reported to produce 40.1 g/L L-threonine, which corresponds to an increase of 83% compared to the wild-type strain MG1655 that had been engineered to carry the same threonine-specific modifications [72, 73]. In separate studies, the consequences of individual deletions or inactivations of genes and operons on L-threonine synthesis were evaluated [3]. In total, 56 genes and operons were identified, which are not involved in L-threonine synthesis but which upon deletion or inactivation led to an increase in L-threonine production. Among the regions deleted are IS elements, toxin–antitoxin pairs, toxins, surface structures, small RNAs, and others. The reason for increased production upon deletion of these genes is unclear. In general, three explanations are possible:

- Genome reduction leads to a decrease in the metabolic burden and the surplus of metabolic power and carbon source is used for product formation.
- Genome reduction has a favorable regulatory influence on the metabolic network.
- Genome reduction including the deletion of IS elements reduces genomic alterations with a negative impact on productivity.

The first two arguments are difficult to assess, in particular since they are mixed. It is well known that even the deletion of single genes can have complex regulatory consequences [74, 75]. Whether there is a correlation between a reduced genome size and increased metabolic power or productivity requires further studies. On more solid grounds is the argument that the absence of IS elements can improve the production properties. In metabolic engineering, producer strains are constructed in a stepwise manner involving genetic manipulations, with each step within this process being prone to the acquisition of unwanted mutations. Since IS elements cause many of the spontaneous mutations occurring in *E. coli* [76], their removal helps to reduce the occurrence of such mutations. Moreover, under producing conditions, a number of factors stress the cells, like osmotic effects, temperature and substrate fluctuations, or oxygen deprivation [2, 77], meaning that in the final high-level producer, again the occurrence of mutations is favored which reduce productivity and thereby also stress. Eliminating stress-inducible error-prone DNA polymerases in an IS element-free strain of *E. coli* showed a close to 50% decrease in the spontaneous mutation rate [76].

First approaches to reduce the genome size in *C. glutamicum* focused on the deletion of strain-specific islands in *C. glutamicum* strain R [78]. Eleven islands representing 250 kb (7.5% of the genome) not present in *C. glutamicum* ATCC13032 were individually deleted, with the resulting strains not influenced in growth. Deletion of genomic fragments of up to 186 kb in *C. glutamicum* R had variable effects on growth [79]. Mutant RMD(190) of *C. glutamicum* R combines deletions corresponding to a total of 190 kb with 188 genes and exhibits no growth defect in minimal medium [78, 80]. *C. glutamicum* ATCC13032 contains three prophages which have been deleted to reduce the genome size by 6% [68]. The resulting strain MB001 did not show any unfavorable properties during extensive

phenotypic characterization under various standard and stress conditions but had an increased transformation efficiency and increased model protein production. The latter property is caused by the deletion of a restriction modification system located on prophage CGP3 [68]. On the basis of strain MB001, 36 individual deletions of up to 50 kb were introduced of which 26 did not affect either the growth rate or the biomass yield in glucose minimal medium [81]. The growth defects caused by some of the ten deletions could be abolished by supplementation of the minimal medium with yeast extract, L-threonine, and vitamins, suggesting defects in anabolism. As a first step toward further genome reduction, five strains were constructed in which two of the deletions without effect on growth in minimal medium were combined. Of these double-deletion strains, three exhibited growth characteristics on minimal medium still comparable to that of the wild type, whereas two showed impaired growth, illustrating that the interactions within the metabolic and regulatory networks of the cell are still unpredictable to a large extent [81].

4.2 Large DNA Fragment Swapping of Useful Traits

In microbial strain development, there is an increasing need to exchange large DNA fragments between genomes and episomes to combine required or desired biological functions of strains or plasmids. For example, the transfer of long biosynthesis pathways from a donor species not suitable for large-scale production to an industrially established microbial host can require such techniques [82]. Manipulation of large fragments within genomes also helps to understand the genome architecture and global gene regulation and consequently growth and production properties [83].

A clear disadvantage of producer strains is reduced sugar consumption and reduced growth. This is frequently the case for strains derived classically by random mutagenesis and screening and a consequence of accumulating mutations during the successive steps of strain development. For example, lysine producer strains of *C. glutamicum* derived in this way are known to contain up to or even more than 1,000 mutations. In an early approach, the genetic properties leading to good L-lysine yields of a *C. glutamicum* strain showing low sugar consumption were combined with a strain showing no productivity but high sugar consumption [84]. This was achieved by protoplast fusion and use of resistance markers characteristic for each strain, leading to a fusion strain with threefold increased glucose uptake and L-lysine production rates. Although a less defined genetic technique was used, this example illustrates well the need to optimize strain properties by novel methods. The efficiency of sugar uptake is also of concern for *E. coli* strains, where rational design was employed for improvement [85]. Strain MG1655 is a popular K-12 strain in use for metabolic engineering but carries *ilvG* and *rph-1* mutations which have a negative impact on growth under certain conditions. Removal of these mutations increased the growth rate substantially from 0.56 to 0.73 h^{-1}

recommending this novel strain as more suitable for small molecule production [86].

The main approaches to integrate fragments into the *E. coli* chromosome involve recombineering [7, 87] and phage-derived methods [88, 89]. However, as mentioned above, recombineering suffers from the size limitation of the DNA fragment when provided as a PCR product, and the risk of unwanted mutations is increased upon the use of larger PCR fragments. To achieve efficient integration of fragments up to 7 kb, a method has been described where the fragment is supplied from a plasmid and the recombineering efficiency is increased by the use of the meganuclease I-SceI, which produces double-strand breaks at a unique 18 bp recognition sequence [24]. The cells contained a helper plasmid harboring genes encoding the λ Red enzymes and the I-SceI endonuclease. In the first step, the λ Red enzymes expressed from the helper plasmid are used to recombineer a small (1.3 kb) "landing pad" into the desired location in the chromosome, consisting of a tetracycline resistance gene flanked on each site by I-SceI endonuclease recognition sites and a defined 25-bp random sequence not present in the *E. coli* genome. In the second step, the cell containing the "landing pad" is transformed with the donor plasmid carrying the desired insertion fragment similarly flanked by I-SceI recognition sites and the same 25-bp random sequences as the "landing pad" region. Induction of I-SceI expression led to cleavage of both the donor plasmid and the chromosome and the successful targeted integration of the 7 kb fragment into the chromosome. The integration is highly efficient, and different fragments can be targeted into the chromosome at separate locations via the repeated use of the universal system developed. The method bears some resemblance to the ALFIRE method for BAC (bacterial artificial chromosome) subcloning [90], which was shown to allow the transfer between BACs of large fragments up to 55 kb in size. It is therefore likely that the use of DSBs generated by I-SceI and the activity of the Red/ET recombinases will allow for the insertion of similarly large fragments into the chromosome using a donor BAC.

An additional approach to the use of I-SceI and recombineering makes use of phage-integration systems to facilitate the insertion of synthetic constructs into the chromosome [88, 89]. Again "landing pads" are used, in this case well known as phage attachment sites *att*. The donor DNA contains a phage-specific attachment site (*attP*), which, when transformed into a host cell expressing the appropriate phage integrase enzyme, is integrated into a complementary phage *attB* attachment site in the chromosome [91]. These systems have the advantage that either the delivery of fragments from plasmids or by transduction is possible and that there is effectively no limit to the size of the fragment that can be inserted at the attachment site. When additionally the appropriate phage Xis enzyme is expressed, the backbone of the donor plasmid can also be easily removed [17, 89]. In one such application, the *attB* attachment site of phage φ80 was integrated at several locations in the *E. coli* chromosome via λ Red recombineering, representing the "landing pad" [92]. Using plasmids carrying the φ80-*attP* site, chromosomal 8 kb fragments excised by the use of their flanking I-SceI sites were successfully integrated by this procedure [93]. Because large chromosomal fragments can be

flanked by I-SceI sites and cloned into vectors [24], significantly larger fragments may be integrated by the use of such a procedure.

One experiment demonstrated the use of large fragment integration to convert the alginate of brown seaweed by *E. coli* into ethanol. The alginate catabolic pathway is present on a 34 kb fragment in *Vibrio splendidus* [94]. In the first engineering step, the "landing pad" was introduced at the desired location into the *E. coli* genome using λ Red recombination. The "landing pad" consisted in this case of *lox* sites flanking an antibiotic marker [95]. The 31 bp *lox* sequence is recognized by the site-specific Cre recombinase of the Cre/lox system derived from phage P1. In the second step, the similarly *lox*-flanked 34 kb fragment was delivered from a single-copy plasmid previously transferred into the recipient strain. Selection for the presence of an antibiotic marker previously introduced into the 34 kb fragment and the absence of the antibiotic marker present on the "landing pad" yielded the desired strain [95]. The additional integration of pyruvate decarboxylase and alcohol dehydrogenase genes enabled *E. coli* BAL 1075 to produce more than 20 g/L ethanol with a productivity of 0.43 g/L/h from brown seaweed demonstrating its use as feedstock for production of biofuels and commodity chemical compounds.

The Cre/lox system operates in a number of biotechnologically relevant organisms like *C. glutamicum* [96, 97], *Lactococcus lactis* [98], or *Bacillus subtilis* [99]. The *lox* attachment site is usually placed into the genome with λ Red, but this can also be done by the use of engineered mobile group II introns, a recently introduced procedure which does not require selectable markers [100]. Such mobile group II introns delivering *lox* sites were developed for *E. coli*, *Staphylococcus aureus*, *B. subtilis*, and *Shewanella oneidensis* [100]. Among other manipulations, the system was used to deliver a 12-kb polyketide synthase operon to the genome of *E. coli*, to move 100 kb of the *E. coli* genome to another locus 1.5 Mb away, and to invert approximately one-third of the *B. subtilis* genome. As expected, not all of these genome rearrangements were stable because the structure of the chromosome limits genome plasticity [83], but the development of tools is in progress which offer new options for analyzing and engineering entire genomes and to assay their usefulness for small molecule production.

Another important whole-genome scale tool is conjugation, which allows the transfer of very large genomic fragments from one strain to another. Together with transduction, it is one of the earliest systems used in *E. coli* for moving genes and genome fragments between strains [7]. In a recent work, 32 different genomic regions (each about 143 kb) in individual *E. coli* strains, together covering the entire genome, were engineered by MAGE to replace all 314 TAG stop codons with TAA stop codons [101]. With the aim to combine all 314 mutations in one single strain, a method termed "conjugative assembly genome engineering" (CAGE) was developed based on conjugative transfer. For this purpose, the mutated region of the donor strain was flanked with the origin of transfer, *oriT*, on one side and a selectable marker on the other side. The mutated region of the recipient strain was flanked by another selectable marker on one side and a positive–negative selection marker (such a *tolC* or *galK*) on the other side. The other transfer

functions were maintained episomally on the F′ factor. The conjugative transfer was done in parallel in a hierarchical process involving five steps. In the first step, 16 strains were generated with two neighboring 143 kb genomic fragments combined, leading to mutated regions with an average size of 267 kb. In the next step, eight strains were produced with a reengineered genome of an average size of 575 kb. This required additional efforts, due to marker instability and the known exponential decrease of marker transfer with increase in its distance from *oriT* [102]. Finally, a strain was obtained in which all 314 TAG stop codons were exchanged and in addition the release factor 1 gene deleted, thus allowing reassignment of the TAG translation function [103]. Conjugation permits the exploration of vast genetic landscapes. It is one step forward in the direction to understand mutations in a classically derived strain which only result in increased strain performance when present in combination.

5 Genetically Encoded Biosensors Have Bright Prospects for Strain Development

According to IUPAC, a biosensor is a device that uses specific biochemical reactions mediated by isolated enzymes, immune system, tissues, organelles, or whole cells to detect chemical compounds usually by electrical, thermal, or optical signals. Of particular interest in strain development are genetically encoded biosensors based on transcriptional regulators (TRs), which in dependence of a certain stimulus control expression of a reporter gene encoding a fluorescent protein. Biosensor-based techniques enable the use of single-cell analysis via fluorescence-activated cell sorting (FACS) and thus ultrahigh-throughput screenings. They provide a new tool for accelerated strain development [1, 104–107] and can be used advantageously to (1) identify new profitable genomic mutations, (2) generate enzyme variants decisive to overcome flux control, (3) generate – combined with recombineering – productive variants at a specific genomic locus, (4) isolate transcription factors with altered specificity, (5) isolate enzyme variants with altered specificity, and (6) detect population heterogeneity.

Metabolic engineering approaches focus mostly on known genes and known mutations. This approach misses the many unknowns involved in the development of excellent production properties, like functions of the host or the interplay of genes introduced. Therefore, screening approaches are still a highly valuable tool in strain development. In fact, also for such a classical product like L-lysine, there is an ongoing effort for strain improvement involving rounds of mutagenesis and screenings [2]. Biosensors operating at the single-cell level offer unique opportunities for accelerated strain engineering. A prominent example of a TR-based biosensor and its purposeful use together with FACS is LysG of *C. glutamicum* (Fig. 3). LysG is a LysR-type TR that activates expression of its target gene *lysE* (encoding a lysine exporter) in response to elevated intracellular concentrations of L-lysine, L-arginine,

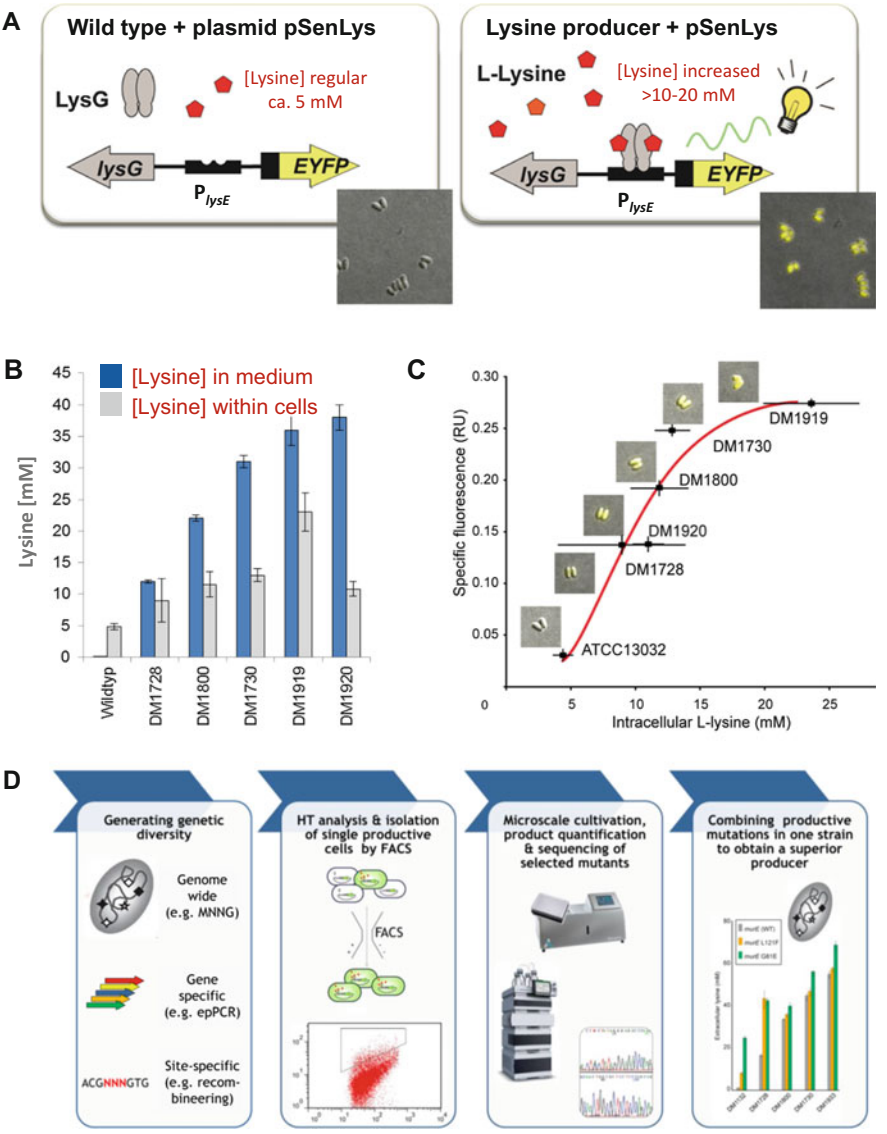

Fig. 3 **A** Basic principle of a metabolite sensor based on a transcriptional regulator. LysG of *C. glutamicum* activates expression of its target promoter P_{lysE} when the cytoplasmic L-lysine concentration surpasses the regular concentration of about 5 mM. This is true, for example, in lysine production strains. On plasmid pSenLys, P_{lysE} controls expression of the reporter gene *eyfp* encoding *yellow* fluorescent protein. Whereas wild-type cells carrying pSenLys are not fluorescent, lysine producer cells with pSenLys are fluorescent. **B** Correlation between intracellular and extracellular L-lysine concentrations in *C. glutamicum* wild type and a series of genetically defined L-lysine producer strains all carrying plasmid pSenLys. **C** Correlation between intracellular L-lysine concentration and specific fluorescence of the *C. glutamicum* strains shown on the left panel. The increased fluorescence is also visible at the single-cell level. **D** Application of metabolite sensors for strain development. Cells equipped with a sensor plasmid for a suitable target metabolite are subjected to mutagenesis either genome wide or gene specific or site specific.

and L-histidine [108]. LysG requires binding of L-lysine, L-arginine, or L-histidine as coactivators in order to activate expression of *lysE* and thus functions as a sensor for the intracellular concentration of basic amino acids with K_d values in the low millimolar range. Using a plasmid containing *eyfp* (encoding enhanced yellow fluorescent protein) as reporter gene under the control of the P_{lysE} promoter, the intracellular L-lysine concentration can be converted into a fluorescence signal. Importantly, increased intracellular concentrations correlate with increased extracellular concentrations [109]. Using the L-lysine responsiveness of LysG, a library of seven million individual cells of *C. glutamicum* obtained by random chemical mutagenesis was subjected to FACS and within 1 h yielded more than 100 L-lysine producers. Their analysis led to the identification of novel productive mutations in previously known target genes, such as *lysC* encoding aspartate kinase. More importantly, whole-genome sequencing identified a novel mutation improving lysine production in a previously unknown target gene, *murE*, encoding an enzyme involved in murein biosynthesis, UDP-*N*-acetylmuramoyl-L-alanyl-D-glutamate-2,6-diaminopimelate ligase. Introduction of the identified mutation *murE*-G81E into the *C. glutamicum* wild type triggered significant L-lysine excretion, and its introduction into an existing L-lysine producer increased L-lysine accumulation by 15% [109]. In a similar study, the TR Lrp of *C. glutamicum* was used to successfully screen for genomic mutants synthesizing increased concentrations of the branched-chain amino acids or of L-methionine [110]. More recently, the Lrp-based biosensor was employed for adaptive laboratory evolution to isolate *C. glutamicum* Δ*aceE* variants with increased growth rates, increased valine titers, and reduced by-product formation. Genome re-sequencing led to the identification of mutations that are responsible for the improved traits [111].

Besides FACS-based screening of cell libraries for productive genomic mutations increasing lysine synthesis, the LysG-based biosensor was also used in a targeted approach to isolate feedback-resistant variants of the key enzymes of L-lysine, L-arginine, and L-histidine biosynthesis. The *N*-acetyl-L-glutamate kinase, ArgB, of *C. glutamicum* exerts flux control over the long L-arginine biosynthesis pathway due to allosteric inhibition of its activity by elevated L-arginine concentrations. A plasmid-based *argB* mutant library was introduced into *C. glutamicum* carrying the LysG sensor, and about 22×10^6 cells of the mutant library were screened via FACS. Ninety-six single cells exhibiting increased fluorescence were selected. Downstream analyses revealed that 41 clonal cultures accumulated up to 18 mM L-arginine and that this was due to ArgB variants which are no longer feedback inhibited by L-arginine [112]. A similar approach was used to obtain collections of feedback-resistant variants of aspartate kinase and ATP

Fig. 3 (continued) The resulting libraries are screened by FACS and highly fluorescent cells are sorted out. These are characterized further with respect to growth, target metabolite overproduction in the medium, and genome sequence. Productive mutations identified by comparative analysis of the genome sequence and further analyses are combined in one strain to obtain a superior producer strain. *MNNG* 1-methyl-3-nitro-1-nitrosoguanidine, *epPCR* error-prone PCR

phosphoribosyltransferase, which are the key enzymes of L-lysine and L-histidine synthesis, respectively [112]. The mutations identified can be assembled with further productive mutations for rapid development of producer strains.

An even further advanced application of biosensor-based FACS screening is its combination with recombineering, a technology called RecFACS [15]. RecFACS enables the high-throughput identification via FACS of productive mutants in a population obtained directly from recombineering assays. Using a mixture of 19 different oligonucleotides to target the codon *murE*-G81 in the chromosome of *C. glutamicum*, in a single RecFACS experiment, a set of mutants was isolated covering 12 different amino acid exchanges at *murE*-G81 all leading to different L-lysine production titers [15]. Thus, RecFACS allowed introduction of genetic diversity and screening for productive mutations in one single step.

In synthetic biology frequently entire new pathways are established which have to be improved. This may require the adjustment of a variety of plasmid-encoded functions as well as functions of the host chromosome. An example where a ribosome-binding site was targeted by the use of a biosensor is mevalonate synthesis with *E. coli* [113]. To achieve this, first a mevalonate-responsive derivative of the arabinose-sensing TR AraC was isolated. The *gfp*uv reporter gene encoding green fluorescent protein (GFPuv) was placed downstream of the AraC-controlled promoter P_{BAD}, and *E. coli* clones expressing an AraC library were screened via FACS for mevalonate responsiveness. After several rounds of screening, AraC-mev became available carrying four amino acid substitutions. AraC-mev exhibited an almost linear response to exogenous mevalonate concentrations in the range of 10–100 mM. An AraC-mev-based biosensor coupled to β-galactosidase expression was used in an *E. coli* strain producing mevalonate due to plasmid-encoded hydroxymethylglutaryl-CoA reductase and two further genes [113]. Libraries of variant ribosome-binding sites in front of the hydroxymethylglutaryl-CoA reductase coding region were successfully screened by colony color for increased mevalonate formation, and a variant responsible for a fourfold increase in the mevalonate titer was isolated. In addition to AraC-mev also AraC-TAL was developed [114]. The latter TR is responsive to triacetic acid lactone (TAL), which is the product of 2-pyrone synthase activity of *Gerbera hybrida* and an intermediate of polyketide synthesis. Plasmid-based libraries of 2-pyrone synthase were introduced into *E. coli* equipped with AraC-TAL coupled to *lacZ* as reporter gene. Two rounds of mutagenesis and visual screening on plates for colony color yielded a variant conferring ∼20-fold increased TAL production due to an increased catalytic efficiency of the mutated 2-pyrone synthase. Another biosensor used the TR BmoR of *Thauera butanivorans* to couple butanol production by *E. coli* to increased tetracycline resistance [115]. A limited 96-well plate screen for variant ribosome-binding sites yielded a pathway variant with 35% increased specific productivity.

As described above, biosensors can be employed for enzyme development by sensing either the direct product of the enzyme or a product further downstream in a pathway whose synthesis is dependent on the enzymatic activity in question [107]. A somewhat different type of biosensor monitors the NADPH demand of

the *E. coli* cell and is consequently a generalizable system enabling screening for high NADPH oxidation activity by enzymes [116]. It circumvents the problem that for each new enzyme development, a new biosensor has to be constructed. The NADPH biosensor is based on the [2Fe–2S] cluster-containing TR SoxR of *E. coli* that activates expression of *soxS* in the oxidized but not in the reduced state [117, 118]. As SoxR is retained in the cell in its reduced state by NADPH-dependent reductases [119], an increased NADPH demand counteracts SoxR reduction and increases *soxS* expression [120]. For measuring transcriptional activation of the *soxS* promoter, it was fused to the reporter gene *eyfp*. When testing this system with an NADPH-dependent alcohol dehydrogenase, which reduces methyl acetoacetate to (*R*)-methyl 3-hydroxybutyrate, a correlation between cellular fluorescence and alcohol dehydrogenase enzyme activity was observed. Therefore, this type of biosensor is suitable for evolving NADPH-dependent dehydrogenases by FACS screening of mutant libraries. The sensor was successfully used to screen an alcohol dehydrogenase library for variants showing improved activity with the substrate 4-methyl-2-pentanone [116].

The examples described above demonstrate the power of biosensor-based FACS screenings for strain and enzyme development. This technology expands the genome engineering techniques substantially. For instance, the fact that they directly deliver producing bacteria will enable to isolate cohorts of strains with similar phenotypic production properties, which together with whole-genome sequencing might unravel so far unknown hot spots in the genome relevant for increased production. Depending on the application, biosensors with high specificity and also with different sensitivities for the same small molecule might be of interest, thus requiring the further development of TRs.

6 Outlook

The approaches described above open up new avenues for microbial strain breeding. Obviously, they need to be integrated with a range of other technologies, such as next-generation sequencing, bioinformatics, and high-throughput phenotyping. The multiplicity of strains generated by the methods described above needs to be characterized and compared for growth and production, requiring automation of bioprocess development [121]. Furthermore, high-throughput analysis of strains by genomics, transcriptomics, proteomics, metabolomics, and fluxomics would be highly desirable to allow a global functional characterization of strains and novel insights into the metabolic and regulatory networks. Suitable bioinformatics tools that intelligently link the results of multi-omics experiments are crucial for evaluation. Another topic closely related to strain breeding is enzyme development. The prediction of the effect of mutations on enzyme activity is still usually impossible, and improved technologies for enzyme development would strongly contribute also to strain development. Although many of the listed technologies still have to be developed or are not yet state of the art, it is likely that microbial strain breeding

will become much faster and more efficient in the near future and thus contribute to the establishment of a sustainable bioeconomy.

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Microbial Production of Amino Acid-Related Compounds

Volker F. Wendisch

Abstract *Corynebacterium glutamicum* is the workhorse of the production of proteinogenic amino acids used in food and feed biotechnology. After more than 50 years of safe amino acid production, *C. glutamicum* has recently also been engineered for the production of amino acid-derived compounds, which find various applications, e.g., as synthons for the chemical industry in several markets including the polymer market. The amino acid-derived compounds such as non-proteinogenic ω-amino acids, α,ω-diamines, and cyclic or hydroxylated amino acids have similar carbon backbones and functional groups as their amino acid precursors. Decarboxylation of amino acids may yield ω-amino acids such as β-alanine, γ-aminobutyrate, and δ-aminovalerate as well as α,ω-diamines such as putrescine and cadaverine. Since transamination is the final step in several amino acid biosynthesis pathways, 2-keto acids as immediate amino acid precursors are also amenable to production using recombinant *C. glutamicum* strains. Approaches for metabolic engineering of *C. glutamicum* for production of amino acid-derived compounds will be described, and where applicable, production from alternative carbon sources or use of genome streamline will be referred to. The excellent large-scale fermentation experience with *C. glutamicum* offers the possibility that these amino acid-derived speciality products may enter large-volume markets.

Keywords 2-Ketoglutarate, 2-Ketoisocaproate, 2-Ketoisovalerate, Beta-alanine, Cadaverine, Diamines, Ectoine, GABA, L-Citrulline, L-Ornithine, Putrescine, Pyruvate, Trans-4-hydroxyproline

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Contents

1 Introduction

As it is used in million-ton-scale amino acid production, *C. glutamicum* has been studied intensely by classical strain improvement [1, 2], traditional metabolic engineering [3–5], systems biology approaches [6, 7], and by synthetic biology approaches [8, 9] aimed at enlarging its substrate [10] and product range [9, 11]. In this chapter, metabolic engineering of *C. glutamicum* for the production of α,ω-diamines, ω-amino acids [12], 2-keto acids, and other non-proteinogenic amino acids is in the focus of the following four sections, while traditional products like endogenous amino acids and organic acids will not be covered.

2 *C. glutamicum* Strains Overproducing 2-Keto Acids

The 2-keto acids pyruvic acid, 2-ketoglutaric acid, 2-ketoisovaleric acid, and 2-ketoisocaproic acid (Fig. 1) are aminated to the amino acids L-alanine, L-glutamate, L-valine, and L-leucine, respectively. *C. glutamicum* strains engineered to overproduce one of these amino acids can be converted to the respective 2-keto acid-producing strains by eliminating or reducing transamination and/or reductive

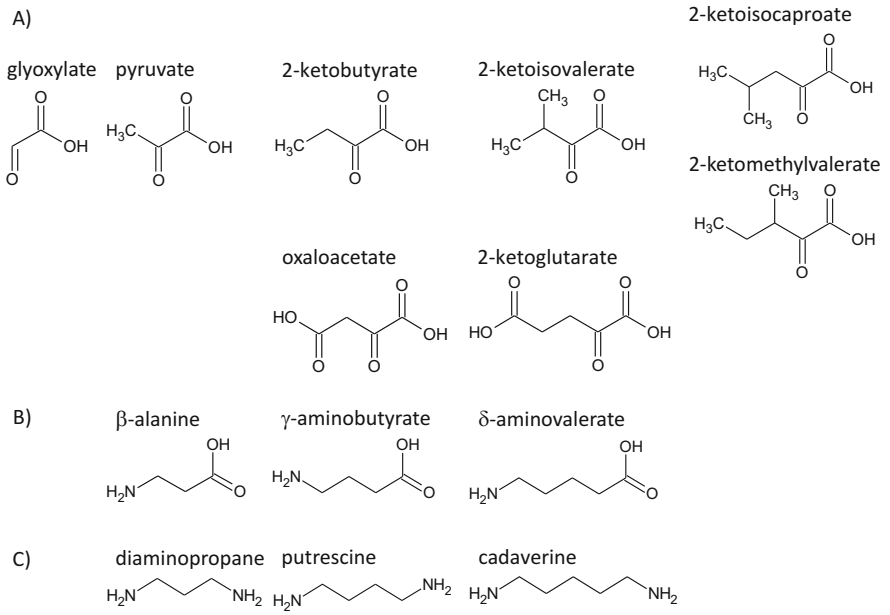

Fig. 1 Structures of 2-keto acids (**a**), ω-amino acids (**b**), and α,ω-diamines (**c**) accessible by fermentation using *C. glutamicum* strains

amination reactions. Fermentative production of the 2-keto acid oxaloacetic acid, precursor of L-aspartate, has not yet been demonstrated. Similarly, fermentative production of the 2-keto acid glyoxylic acid, precursor of L-glycine in some bacteria, has not yet been reported; however, *C. glutamicum* has been engineered to overproduce glycolate [13], which is formed by reduction of glyoxylic acid.

2.1 Pyruvate

Pyruvic acid is one of the central intermediates of carbon metabolism. Pyruvic acid is used in food, cosmetic, and pharmaceutical industries. Compounds derived from pyruvate are often excreted, but pyruvic acid itself typically is not excreted under these overflow conditions. Under oxygen-deprivation conditions, for example, pyruvate is reduced to lactic acid, which is excreted. Pyruvate can be converted to acetate, which is excreted. Since in these reactions either NAD^+ is regenerated by lactate dehydrogenase or ATP is formed by acetate kinase, lactate and acetate excretions are favored over pyruvate excretion. To convert *C. glutamicum* to an efficient pyruvate producer, the genes for pyruvate dehydrogenase (*aceE*), pyruvate: quinone oxidoreductase (*pqo*), lactate dehydrogenase (*ldhA*), alanine aminotransferase (*alaT*), and valine-pyruvate aminotransferase (*avtA*) as well as part of acetohydroxyacid synthase (AHAS) gene (ΔC-T *ilvN*) were deleted to avoid

conversion to acetyl-CoA, acetic acid, lactic acid, and alanine. Titers of more than 45 g/l pyruvic acid were achieved using this strain [14].

2.2 2-Ketoglutarate

2-Ketoglutaric acid finds applications in the pharmaceutical and food industries. 2-Ketoglutarate is a branch point metabolite of the tricarboxylic acid (TCA) cycle and immediate precursor of L-glutamate. To prevent amination of 2-ketoglutaric acid to L-glutamate, two pathways had to be deleted. On the one hand, glutamate dehydrogenase catalyzes NADPH-dependent reductive amination of 2-ketoglutarate, and, on the other hand, the combined reactions of glutamine-2-oxoglutarate aminotransferase (GOGAT) and glutamine synthetase convert 2-ketoglutarate to L-glutamate requiring ATP and NADPH. Deletions of the respective genes, *gdh* and *gltB*, resulted in secretion of 2-ketoglutaric acid into the medium [15]. Degradation of isocitrate, the direct precursor of 2-ketoglutaric acid, was decreased by deletion of *aceA*, which encodes the key enzyme of the glyoxylate bypass shunt, isocitrate lyase. The resulting strain was auxotrophic for L-glutamate and accumulated 2-ketoglutarate to titers of about 12 g/l [15].

2.3 2-Ketoisovalerate

2-Ketoisovaleric acid is a precursor for drugs used as treatment against kidney disease. L-Valine is synthesized from pyruvic acid via acetohydroxyacid synthase (AHAS, encoded by *ilvBN*), acetohydroxyacid isomeroreductase (AHAIR, encoded by *ilvC*), dihydroxy-acid dehydratase (DHAD encoded by *ilvD*) and transaminase B (encoded by *ilvE*). To prevent L-valine formation, *ilvE* was deleted. Efficient excretion of 2-ketoisovalerate required *ilvBNCD* overexpression and prevention of acetate formation from pyruvate by deletion of *pqo* (encoding pyruvate quinone oxidoreductase) and *aceE* (encoding a subunit of pyruvate dehydrogenase) [16]. Subsequently, it was shown that instead of deleting *aceE*, leaky *aceE* expression from a weak promoter resulted in 2-ketoisovalerate titer of 35 g/l, a product yield of 0.24 mol per mol of glucose and a volumetric productivity of 0.8 g/(l h), when also *ppc* gene encoding PEP carboxylase was deleted [17]. The conversion of strains optimized for L-valine production under oxygen-deprivation conditions [18] has not yet been described.

2.4 2-Ketoisocaproate

2-Ketoisocaproate has industrial and medical applications including its use as therapeutic against of chronic kidney disease and to modulate insulin and glucagon release. *C. glutamicum* strains accumulated 2-ketoisocaproate as consequence of deletion or reduced expression of the *ilvE* gene for transaminase B [19, 20], deletion of *lbtR* encoding the leucine and tryptophan biosynthesis repressor [21] and overexpression of isoleucine and leucine biosynthesis genes as well as employing feedback-resistant 2-isopropylmalate synthase and acetohydroxyacid synthase. Moreover, deletion of the regulatory gene *iolR* [22] or methylcitrate synthase genes [23] combined with leaky expression of citrate synthase gene *gltA* [24] improved 2-ketoisocaproate production [19, 20]. The engineered strains accumulated up to about 9 g/l 2-ketoisocaproate and product yields of 0.20 mol per mole of glucose and 0.24 mol carbon in 2-ketoisocaproate per mole carbon in a glucose-acetate mixture were achieved. Investigation of the activities of the native isopropylmalate synthase revealed that this enzyme is not only feedback inhibited by leucine (K_i of around 0.05 mM) but also by 2-ketoisocaproate in a competitive manner (K_i of around 8 mM) [19]. Moreover, isopropylmalate dehydratase was shown to be noncompetitively inhibited by 2-ketoisocaproate (K_i of around 5 mM), while leucine did not affect isopropylmalate dehydratase activity [19].

2.5 Conversion of 2-Keto Acids to Alcohols

A number of higher alcohols are accessible by decarboxylation and subsequent reduction of 2-keto acids [25, 26]. These alcohols are superior to ethanol since they exhibit higher energy density, lower corrosiveness, vapor pressure, and hygroscopicity and are compatible with existing fuel infrastructure [27]. Decarboxylation of 2-keto acids such as 2-ketoisovalerate or 2-ketoisocaproate may be catalyzed by broad-range, 2-ketoacid decarboxylases such as KivD from *Lactococcus lactis* [28]. Alcohol dehydrogenase 2 of *S. cerevisiae* may be used for the subsequent reduction to the respective alcohols [28]. Also *C. glutamicum* has been engineered for isobutanol production by decarboxylation and reduction of 2-ketoisovalerate [29, 30]. Besides heterologous expression of *kivD* from *Lactococcus lactis*, the gene for the native alcohol dehydrogenase gene (*adhA*) was overexpressed [29]. Strain improvements included improved NADPH generation by overexpression of *pntAB* from *E. coli* encoding transhydrogenase and by deletion of fermentative NAD-dependent, L-lactate dehydrogenase gene *ldhA*. The achieved titer of about 13 g/l isobutanol [29] was in the same range as those obtained using recombinant *E. coli* and *B. subtilis* [31]. *C. glutamicum* has not yet been engineered for production of other alcohols derived from 2-keto acids except ethanol [32] and isobutanol.

2.6 Whole Cell Biotransformation Yielding Oxo-Functionalized Long Chain Fatty Acids

Keto-functionalized fatty acids find application in the plasticizers, lubricants, detergents, cosmetics, and surfactants industries. Recently, whole-cell biotransformation processes using permeabilized *C. glutamicum* cells were described. A *C. glutamicum* strain expressing a secondary alcohol dehydrogenase gene from *Micrococcus luteus* WIUJH20 converted 5 mM ricinoleic acid to 12-ketooleic acid with a molar yield of 70% conversion efficiency [33]. As a follow-up, a derived *C. glutamicum* strain additionally expressing gene *ohyA* encoding oleate hydratase from *Stenotrophomonas maltophilia*, 9 mM oleic acid, were converted to 10-ketostearic acid with about a molar yield of 74% conversion efficiency [34].

3 *C. glutamicum* Strains Overproducing α,ω-Diamines

C. glutamicum has been engineered for the production of α,ω-diamines with carbon chain lengths of 4 and 5, respectively (Fig. 1). These diamines are named putrescine or 1,4-diaminobutane and cadaverine or 1,5-diaminopentane, and they may be used as monomeric precursors of polyamides in condensation reactions with α,ω-dicarboxylic acids [35]. Thus, sustainable production of polyamides is feasible when α,ω-dicarboxylic acids and α,ω-diamines are produced from renewables and provided as drop-in compounds for the established polyamide condensation chemistry.

3.1 Putrescine

The biogenic polyamide precursor putrescine is synthesized either by decarboxylation and deamination of arginine or by direct decarboxylation of ornithine [35]. *C. glutamicum* has been engineered for the production of putrescine from [36] glucose and from alternative carbon sources including pentoses, hemicellulosic hydrolysates [12], hexuronic acids [37], crude glycerol [38], glucosamine [39], and N-acetylglucosamine [40]. Putrescine production by decarboxylation of L-ornithine using *E. coli* ornithine decarboxylase gene *speC* was superior to putrescine production by decarboxylation and deamination of arginine. Derepression of L-arginine biosynthesis by deletion of *argR* combined with leaky expression of *argF* from an addiction plasmid, overexpression of *argB* encoding feedback-resistant N-acetylglutamate kinase ensured efficient and growth-decoupled putrescine production [41]. The use of an addiction plasmid made the addition of antibiotics to the fermentation broth dispensable [41]. Diamines including putrescine [42] and cadaverine [43] were shown to be acetylated by SnaA, a

spermi(di)ne N-acetyltransferase identified in a systematic genetic screen. SnaA showed the highest catalytic efficiency for acylation of the triamine spermidine and the tetraamine spermine with the donors acetyl-CoA or propionyl-CoA [42]. Deletion of *snaA* prevented acetylputrescine formation and improved putrescine production. Overexpression of the putative transport gene *cgmA* increased putrescine production except in strains lacking *snaA* [42]. This gene was identified in a transcriptomics approach, and subsequently the TetR-family transcriptional repressor CgmR that is encoded in an operon with *cgmA* was shown to regulate transcription of *cgmAR* in response to putrescine and other diamines at physiologically relevant concentrations [42]. Based on findings from genome-scale stoichiometric modeling, glycolysis and anaplerosis were enhanced by plasmid-borne overexpression of the genes for glyceraldehyde 3-phosphate dehydrogenase and pyruvate carboxylase [44]. Moreover, 2-ketoglutarate dehydrogenase activity was attenuated by changing the translational start codon of the chromosomal gene for 2-oxoglutarate dehydrogenase subunit E1o to the less preferred TTG and by changing threonine 15 of OdhI to alanine. Currently, the most advanced *C. glutamicum* strain obtained by systems metabolic engineering produces 58.1 ± 0.2 mM with a specific productivity of 0.045 g/(g h) and a yield on glucose of 0.26 g/g [44].

3.2 Cadaverine

Cadaverine is derived of L-lysine by decarboxylation. Mimitsuka et al. were the first to show that cadaverine production resulted when the L-lysine decarboxylase gene *cadA* from *E. coli* was expressed in an L-lysine-producing *C. glutamicum* strain [45]. Since the cadaverine market is potentially large, also *E. coli* [46, 47] and *Bacillus methanolicus* [48] have been engineered for cadaverine production. Moreover, cadaverine can be produced from alternative carbon sources like pentoses [49], starch [50], and methanol [48]. Acetylation of cadaverine was abrogated by deletion of *snaA* [43], which encodes spermi(di)ne N-acetyltransferase [42]. Transport engineering improved cadaverine production when the cadaverine-lysine antiporter gene *cadB* from *E. coli* [51] or endogenous *cgmA* were overexpressed [8]. The most advanced strain produced cadaverine with a molar yield of 50%, a productivity of 2.2 g/(l h) and a final titer of 88 g/l [8]. Pure diaminopentane (99.8%) could be isolated and used for polycondensation with sebacic acid to yield PA5.10. Mechanical properties of this polyamide were at least comparable to the petrochemical polymers, PA6 and PA6.6 [8].

4 *C. glutamicum* Strains Overproducing ω-Amino Acids

Besides the proteinogenic α-amino acids, several ω-amino acids occur as intermediates of cellular metabolism. The ω-amino acids β-alanine and γ-aminobutyric acid (GABA) are derived from L-aspartate and L-glutamate, respectively, by decarboxylation (Fig. 1). L-Lysine can be converted to δ-aminovalerate involving a monooxygenase and a transaminase reaction.

4.1 β-Alanine

β-Alanine is used as synthon in pharmaceutical industry. In *C. glutamicum*, the ω-amino acid β-alanine is synthesized by decarboxylation of L-aspartate and is an intermediate of pantothenic acid biosynthesis. Whereas pantothenate production by recombinant *C. glutamicum* strains has been shown [52], fermentative production of β-alanine has not yet been described. However, the *C. glutamicum* L-aspartate α-decarboxylase gene *panD* [53] has been employed in whole-cell biotransformation of L-aspartate to β-alanine using recombinant *E. coli* [54].

4.2 GABA (γ-Aminobutyric Acid)

The non-proteinogenic ω-amino acid GABA is of relevance in the pharmaceutical industry as it is an inhibitory neurotransmitter of the mammalian nervous system. GABA is also used in functional foods and can be used to produce polyamide 4 by ring-opening polycondensation after conversion to the respective lactam. Heterologous expression of glutamate decarboxylase genes from *E. coli* or lactobacilli resulted in GABA production [55–57]. Since GABA is a carbon source for *C. glutamicum* [58], deletion of the GABA uptake gene prevented GABA reuptake [56–58]. The small inhibitory protein OdhI specifically inhibits 2-ketoglutarate dehydrogenase [59] unless it is phosphorylated by a protein kinase such as PknG [60]. Disruption of *pknG* resulted in reduced 2-ketoglutarate dehydrogenase activity and, thus, improved supply of L-glutamate for GABA production [61].

4.3 δ-Aminovalerate

δ-Aminovalerate is a monomeric precursor of nylon 5. Via the intermediate valerolactam, the polyamide nylon 5 is produced in a ring-opening polycondensation reaction. In cellular metabolism, δ-aminovalerate can be derived from L-lysine via two enzymatic reactions. While the production of δ-aminovalerate has not yet

been shown for *C. glutamicum*, recombinant *E. coli* strains have been engineered for δ-aminovalerate production. L-Lysine-overproducing strains that carried feedback-resistant versions of aspartate kinase III and dihydrodipicolinate synthase and did not produce cadaverine as by-product due to deletion of lysine decarboxylase genes *cadA* and *ldcC* produced δ-aminovalerate when *Pseudomonas putida* genes *davAB* encoding δ-aminovaleramidase and lysine 2-monooxygenase were expressed [62, 63]. As an extension to this concept, the production of glutarate, an α,ω-dicarboxylic acid used as a monomeric precursor of polyamines in condensation reactions with α,ω-diamines, could be established by additional heterologous expression of *P. putida gabTD* genes encoding δ-aminovalerate aminotransferase and glutarate semialdehyde dehydrogenase. For the conversion of lysine to glutarate, the external addition of stoichiometric concentrations of 2-ketoglutarate was required since the latter is substrate of the δ-aminovalerate aminotransferase reaction. While the proof of concept was achieved, titers for δ-aminovaleric and glutarate did not exceed 2 g/l [62, 63].

5 *C. glutamicum* Strains Overproducing Further Non-Proteinogenic Amino Acids

Strains for the production of the non-proteinogenic amino acids L-ornithine and L-citrulline, which are intermediates of L-arginine biosynthesis, of hydroxylated amino acids such as trans-4-hydroxyproline, of cyclic amino acids such as ectoine, and of the D-isomers of a number of amino acids, have been engineered.

5.1 L-Ornithine

L-Ornithine is a potential treatment of liver diseases [64], and as an intermediate of L-arginine biosynthesis, L-arginine-producing strains can be engineered for L-ornithine production. Deletion of *argF* avoided conversion of L-ornithine toward L-arginine by L-ornithine carbamoyltransferase and resulted in about 25 g/l L-ornithine produced from glucose in 72 h using glucose [65]. Increased L-glutamate availability improved L-ornithine production [66]. L-Ornithine can be produced from glycerol [38], pentoses [67], and sucrose [68]. A number of overexpression targets have been identified: *ppnK* [69], which encodes polyphosphate-dependent NAD kinase [70]; *Clostridium acetobutylicum gapC*, which encodes NADP-dependent glyceraldehyde-3-phosphate dehydrogenase [71]; *pgk*, which encodes endogenous 3-phosphoglycerate kinase [72]; *Bacillus subtilis rocG*, which encodes NAD-dependent glutamate dehydrogenase [71]; pentose phosphate pathway genes *pgi*, *zwf*, and *tkt* [73]; and NCgl0462 encoding a putative aminotransferase [74]. Moreover, besides deletion of *argR*, deletion of *proB*, *argF* [73], and of

three genes (NCgl0281, NCgl2582, and NCgl2053) encoding putative $NADP^+$-dependent oxidoreductases [75] improved L-ornithine production.

5.2 L-Citrulline

L-Citrulline can be used as pharmaconutrient since in mammalians it is transferred to the blood stream after ingestion and, thus, can be used as a precursor of L-arginine after conversion to L-arginine in the kidney. L-Citrulline is an intermediate of L-arginine biosynthesis, and an L-arginine-producing *C. glutamicum* strain was engineered to produce L-citrulline as a major product [76]. Deletion of the argininosuccinate synthetase gene *argG* blocked conversion of L-citrulline to L-arginine. The pathway leading to L-citrulline was derepressed by deletion of the arginine repressor gene *argR* and by overexpression of *argF*, encoding L-ornithine carbamoylphosphate transferase, and $argB^{fbr}$, encoding a feedback-resistant variant of N-acetyl L-glutamate kinase. L-Citrulline production from glucose with a yield of 0.38 ± 0.01 g/g and a volumetric productivity of 0.32 ± 0.01 g/(l h) was achieved, and L-citrulline production from starch, xylose, and glucosamine could also be shown [76].

5.3 Hydroxylated Amino Acids

Hydroxylation of L-proline by 2-ketoglutarate-dependent L-proline oxygenase yields trans-4-hydroxyproline, a component of collagen, e.g., used to enhance procollagen synthesis or as chiral synthon for anti-inflammatory drugs. The precursor L-proline, which is used in animal feed and as chemical synthon, can be synthesized from L-glutamate or alternatively from L-ornithine by ornithine cyclodeaminase, which is found in plants, animals, and bacteria such as *Pseudomonas putida*. Upon heterologous expression of the *P. putida* ornithine cyclodeaminase gene, an L-ornithine-producing strain was converted to an L-proline-producing strain accumulating L-proline with a yield of 0.36 g L-proline per g glucose [77]. When in *E. coli* or *C. glutamicum* a gene for L-proline-4-hydroxylase, e.g., from *Dactylosporangium* sp., was expressed, production of trans-4-hydroxyproline resulted without the requirement to add its substrates L-proline or 2-ketoglutarate to the medium [78, 79]. However, much higher titers (up to 7 g/l) were achieved when an L-isoleucine bradytrophic L-proline-overproducing *C. glutamicum* strain was used, and feeding of the carbon substrate glucose and the supplement L-isoleucine was optimized [78]. For hydroxylation of L-isoleucine to yield 4-hydroxyisoleucine, which is used as insulinotropic drug, the gene encoding L-isoleucine dioxygenase from *Bacillus thuringiensis* was heterologously expressed in an L-isoleucine-producing *C. glutamicum* strain [79].

5.4 Cyclic Amino Acids

The cyclic amino acid ectoine ((*S*)-2-methyl-3,4,5,6-tetrahydropyrimidine-4-carboxylic acid) is an osmo-compatible solute used as cell-protective agent in allergic rhinitis and conjunctivitis or atopic dermatitis. Expression of the ectoine biosynthesis operon *ectABCD* from *Pseudomonas stutzeri* in an L-lysine-producing strain and disrupting genes encoding diaminopimelate dehydrogenase and the L-lysine exporter resulted in ectoine production 4.5 g/l [80].

5.5 D-Amino Acids

Besides the proteinogenic L-amino acids, D-amino acids are also found in nature, e.g., in bacterial cell walls or in antibiotics. D-Amino acids may be used as building blocks in the pharmaceutical industry. *C. glutamicum*, which was found to be relatively insensitive to D-amino acids, produced mixtures of D- and L-amino acids when the amino acid racemase gene from *Pseudomonas taetrolens* was overexpressed in the respective L-amino acid-producer strains [81].

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Part IV
Future Perspective of Amino Acid Fermentation

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New Functions and Potential Applications of Amino Acids

Hisayuki Uneyama, Hisamine Kobayashi, and Naoto Tonouchi

Abstract Currently, several types of amino acids are being produced and used worldwide. Nevertheless, several new functions of amino acids have been recently discovered that could result in other applications. For example, oral stimulation by glutamate triggers the cephalic phase response to prepare for food digestion. Further, the stomach and intestines have specific glutamate-recognizing systems in their epithelial mucosa. Regarding clinical applications, addition of monosodium glutamate to the medicinal diet has been shown to markedly enhance gastric secretion in a vagus-dependent manner. Branched-chain amino acids (BCAAs) are the major components of muscles, and ingestion of BCAAs has been found to be effective for decreasing muscle pain. BCAAs are expected to be a solution for the serious issue of aging. Further, ingestion of specific amino acids could be beneficial. Glycine can be ingested for good night's sleep: glycine ingestion before bedtime significantly improved subjective sleep quality. Ingestion of alanine and glutamine effectively accelerates alcohol metabolism, and ingestion of cystine and theanine effectively prevents colds. Finally, amino acids could be used in a novel clinical diagnostic method: the balance of amino acids in the blood could be an

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indicator of the risk of diseases such as cancer. These newly discovered functions of amino acids are expected to contribute to the resolution of various issues.

Keywords Alcohol metabolism, Branched-chain amino acids (BCAAs), Diagnostic indication, Good night's sleep, Prevent cold, Protein digestion, Sarcopenia

Contents

1 Introduction

Amino acid fermentation began with the production of glutamate for use in seasonings. Nevertheless, investigation of the properties of amino acids has resulted in the discovery of several functions that are useful for various fields, such as animal nutrition, human health, and the discovery of substrates for sweeteners or other compounds. Currently, several types of amino acids are being produced in large amounts by fermentation and used worldwide.

Recent research on the function of amino acids has revealed various possible applications and potential demands in the future. For example, glutamate was simply thought to be a compound with taste; however, it has now been revealed that glutamate is sensed in the taste receptors of the gastrointestinal system and the tongue, and it modulates protein digestion. In addition, branched-chain amino acids (BCAAs) have recently received much attention in the healthcare field; they are the major components of muscles. Ingestion of BCAAs has been found to be effective for decreasing muscle pain. Furthermore, BCAAs are expected to be a solution for

the serious issue of "aging" by preventing the degradation of motor skills in the elderly. In this chapter, several topics including newly discovered functions and potential fields for the use of amino acids are described.

2 Glutamate: Umami Is More Than Just a Taste

In 1866, German chemist Ritthausen discovered a new amino acid in wheat gluten hydrolysates and named this amino acid glutamic acid. Glutamate is the most abundant amino acid in nature. Interestingly, it is abundant in human breast milk [1]. So, it is suggested that glutamate must be involved in a wide variety of functions essential for life.

The physiological functions of dietary free glutamate and its nutritional characteristics have been recently discovered. The physiological actions of glutamate are summarized in Fig. 1 [2, 3]. First, glutamate is sensed by the tongue (taste sense), and this triggers preparations for the digestion of protein-containing foods via cephalic phase responses from the oral cavity to the gut (taste-vagal reflex). Then, after swallowing, the glutamate is sensed again in the gut, where it enhances additional gut digestive processes through the visceral sense (vago-vagal reflex). This mechanism could explain the traditional observation that laboratory rats fed on protein-rich diets preferred monosodium glutamate (MSG)-containing solutions [4].

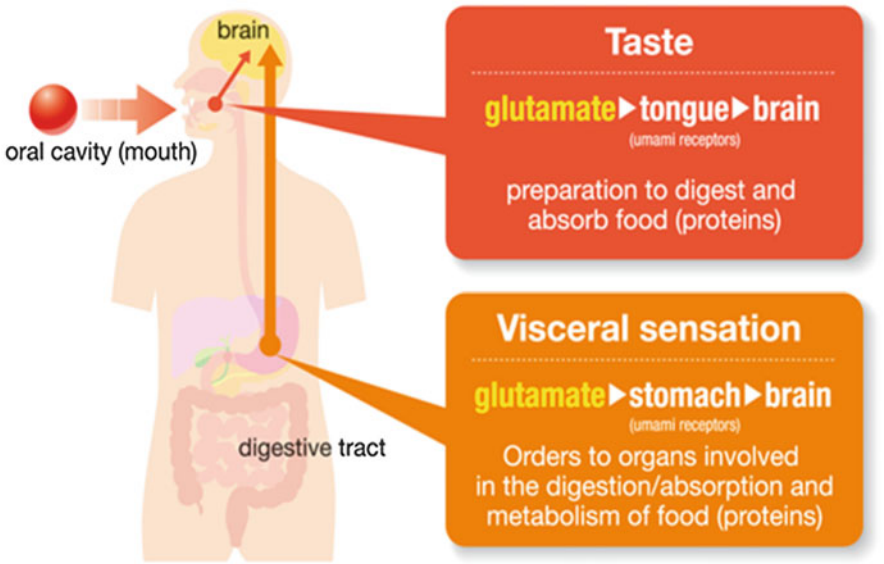

Fig. 1 Mechanism of the physiological role of dietary glutamate in food digestion. Dietary glutamate is sensed throughout the gastrointestinal tract, from the oral cavity to the intestine. These glutamate perception mechanisms might ensure the optimal digestion of dietary protein

2.1 Nutritional Characteristics

Glutamate is one of the nonessential (dispensable) amino acids, but it is expected to have high nutritional efficacy during animal growth. Its nutritional value is higher than that of other nonessential amino acids [5].

The characteristics of glutamate absorption in the intestine are unique. It has been reported that uptake of dietary free glutamate in the pig portal vein is less than 5%; most of the luminal glutamate is metabolized to other amino acids, such as alanine, and utilized as a source of energy within intestinal epithelial cells [6]. Approximately 35% of the total energy consumption of intestinal mucosal cells is derived from dietary glutamate in foods. Thus, glutamate is very important nutrient in the maintenance of intestinal mucosal activity. In addition, glutamate intake has been shown to enhance gut immunity after chemotherapy in rats [7], and enteral nutrition with glutamate effectively enhances the maintenance of intestinal mucosal functions, compared with glutamine [8]. The nutritional benefits of glutamate as a gut essential nutrient should be reconsidered for a healthier life. From this point of view, the nutritional aspects of glutamate are well documented in available reviews [9, 10].

2.2 Taste (Oral Sensation)

The first action of dietary glutamate is the induction of taste (umami) in the oral cavity. Taste is an important sense for the selection of nutritional quality. The sense of taste is thought to have a nutritional and physiological meaning for life. Sweet taste is the signal for energy intake (glucose); salty and bitter tastes are markers of minerals (NaCl) and organic acids, respectively. Umami taste is the signal for protein intake to recruit amino acids essential for life. Recent studies indicate that umami taste substances are food-derived factors that modulate protein digestion. Binding of glutamate to the oral umami taste receptor induces the sense of the taste of glutamate, called umami. Metabotropic glutamate receptors (mGluR1/mGluR3) and amino acid taste receptors (T1R1/T1R3) are now candidates for the umami taste receptor [11].

Oral stimulation of the umami taste also triggers the cephalic phase response, a series of autonomic reflexes, such as salivation related to food mastication/swallowing, to prepare for food digestion [12]. For instance, MSG aqueous solution induces long-lasting and synergic enhancement, with the nucleotide umami taste substance 5′-inosine monophosphate (IMP), of human salivation [13]. In the case of the cephalic phase response, it has already been reported that application of MSG to the oral cavity increases the efferent nerve activities of the abdominal vagus (gastric, celiac, and pancreatic branches) in rats and induces gastric, pancreatic exocrine, and insulin secretions in dogs [14, 15]. Thus, the sense of taste (umami taste) contributes to preparations for food digestion in the gut, as well as efficient

food mastication/swallowing in the oral cavity by initiating exocrine reflexes (saliva, gastric, and pancreatic juices).

2.3 *Visceral Sensation*

After gastric and intestinal digestion, dietary protein is absorbed by enterocytes as peptides and amino acids. Food digestion proceeds efficiently through neuronal (the vagus nerve) and hormonal (gastrointestinal hormones) regulation. Recently, some reports have indicated the possible involvement of gastrointestinal taste receptors in food digestion and nutrient metabolism in the body [16].

The stomach and intestine have specific glutamate recognizing systems in the epithelial mucosa [2, 17]. Umami receptors exist in the stomach and intestine and on the tongue. This physiological aspect is important. Some patients who have undergone throat operations cannot eat food via their mouths. A tube is placed in the throat or stomach, and nutrients are fed directly into the stomach or intestine. Actually, intragastric and duodenal injections activate the afferent nerve activities of the abdominal vagus (Fig. 2).

Interestingly, animal studies have suggested that the ability of the stomach to sense proteogenic amino acids by vagus recognition is different than that of the intestine. The intestine could sense all of the proteogenic amino acids, but the stomach's sensory ability was relatively selective for glutamate [18, 19]. In addition

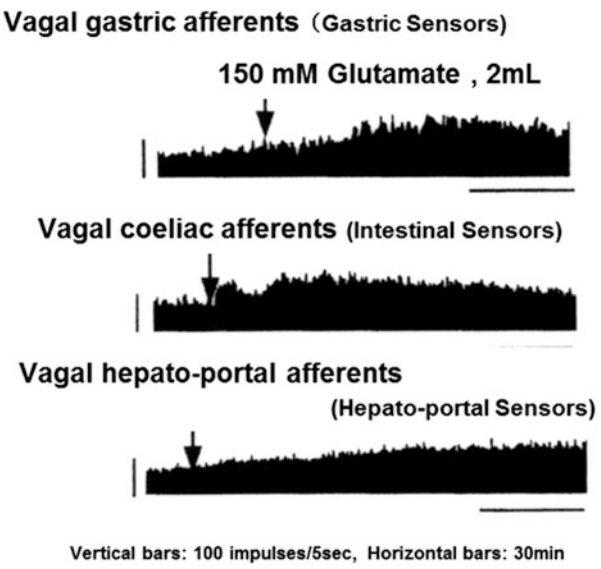

Fig. 2 The effect of monosodium glutamate (MSG) administration on the afferent activities of vagal gastric, intestinal, and hepatoportal glutamate sensors. When food with umami taste was added, neural signals were observed. Modified from [19]

to its neuronal action, the ability of glutamate to stimulate hormones, such as somatostatin, has been preliminarily reported [20].

Because glutamate typically exerts its physiological functions in the gut, regulation of gut exocrine function and motility has been investigated. These investigations have revealed that MSG addition to a glutamate-free medicinal diet (Elental™) markedly enhanced gastric secretions (gastric acid, pepsinogen, and gastric fluid) in dogs in a vagus-dependent manner [21]. Furthermore, MSG fortification of dog food also enhances gastrointestinal movements in dogs [22]. Interestingly, the gastrointestinal actions of MSG might be influenced by the coexistence of macronutrients. A protein diet more effectively supports the MSG effect than a carbohydrate diet [23]. At the same time, the secretion of gut mucosal protective factors (mucin and bicarbonate) is also enhanced by glutamate in rats [24]. Glutamate might maintain the optimal balance between attacking and protective factors. This evidence supports the hypothesis that glutamate in the gut may enhance the digestion of dietary protein by triggering the visceral sense, leading to improved amino acid absorption and utilization.

2.4 Clinical Applications of Glutamic Acid

Several clinical trials have been registered based on the nutritional and physiological actions of glutamate. The oral action of glutamate (umami taste sensation) has the potential to treat oral disorders such as taste disorders and hyposalivation and to improve taste quality, pleasantness, and mastication/swallowing during eating [25–27].

Concerning the visceral sense of glutamate, medicinal diets containing glutamate have been developed for percutaneous endoscopic gastrostomy (PEG) patients in Japan [28]. Chronic gastritis in elderly inpatients is one of problems that reduce appetite and cause protein-energy malnutrition (PEM) through poor gastric digestion. MSG fortification of hospital meals is expected to improve the nutritional status and quality of life (QOL) of elderly inpatients [27]. Adequate usage of glutamate in medicinal/nursing care diets might be a powerful tool to improve the management of inpatient nutrition through stimulating gut functions that lead to optimal protein digestion.

2.5 Functions of Intrinsic Glutamate

In addition to its dietary functions, glutamate has other physiological functions as an intrinsic substance. The most famous physiological function of intrinsic glutamate is its action as an excitatory neurotransmitter in the brain. In particular, more than 80% of excitatory synapses communicate using glutamate. These synapses

participate in basic brain functions such as recognition, learning, and memory formation. In addition to its action in the brain, glutamatergic communication exists in peripheral organs, such as the alimentary tract, the pancreas, and the bone [29, 30].

In clinical applications targeting the actions of intrinsic glutamate, various pharmaceutical approaches focusing mainly on brain glutamate receptors as therapeutic targets have been tried in memory- and psychiatric-related diseases [31]. Glutamate itself was used as an active ingredient of new medicines targeting diseases such as functional dyspepsia and myocardial infarction [32, 33].

3 Branched-Chain Amino Acids: Maintenance of the Muscle and Stamina During Exercise

Leucine, valine, and isoleucine, which contain branched-side chains in their molecular structure, are called branched-chain amino acids (BCAAs). BCAAs are essential amino acids, and their intake improves the nutritional status. Nevertheless, BCAAs, particularly leucine, have functions beyond the role of essential amino acids, i.e., promotion of protein synthesis.

3.1 Sports and Branched-Chain Amino Acids

When strenuous exercise is performed, the body begins to break down proteins and utilize BCAA reserves to compensate for insufficient energy sources. In fact, after engaging in strenuous sports, such as running a marathon and cross-country skiing, the post-competition blood level of BCAA levels may be decreased by 20% because of intramuscular BCAA consumption. This fact highlights that during strenuous exercise, the body breaks down muscle tissues, resulting in their damage.

However, muscle damage can be reduced, and lowering of muscular strength can be inhibited by timely replenishment of BCAAs before or during the sport activity. The sufficiently supplied BCAAs are used as an energy source during exercise and create room for energy sources, allowing maintenance of stamina for a long period. Further, if BCAAs are immediately replenished after the sport activity, the damaged muscles will recover promptly, and symptoms such as muscle soreness can be prevented.

In a randomized controlled trial [34], women between 20 and 25 years did squats, specifically seven sets of 20 reps/set. Half of the participants took some BCAAs before working out. The group that took BCAAs experienced lesser muscle pain than the group that did not take BCAAs (Fig. 3). Therefore, muscle pain was reduced by taking BCAAs before the workout.

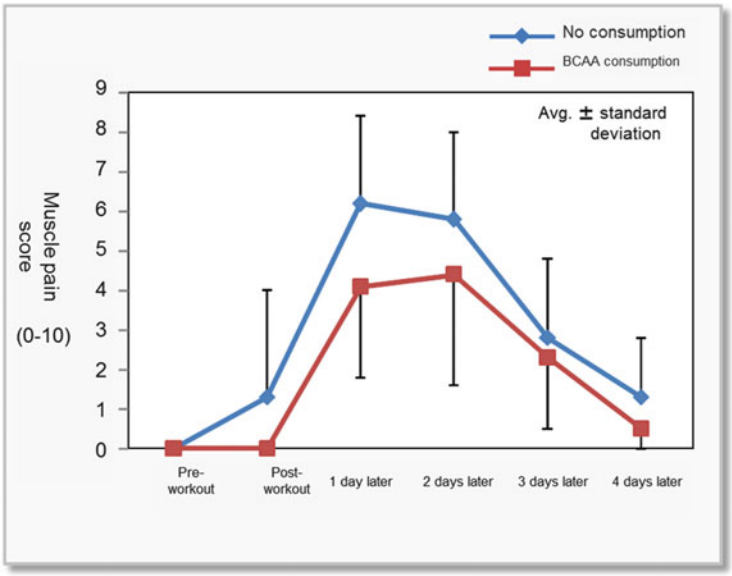

Fig. 3 BCAA intake alleviates post-workout muscle pain. Methodology: The subjects were women with normal health (ages 20–25 years). Amino acid consumption was 100 mg per kg bodyweight. The exercise performed was squats (seven sets of 20 reps/set). Muscle pain was measured pre- and post-workout as labeled on the graph. Modified from [34]

BCAA supplementation before or after exercise improves recovery of damaged muscles.

3.2 Branched-Chain Amino Acids as Regulatory Molecules

The three BCAAs are dietary essential amino acids that play a variety of roles in the body. However, BCAAs are not just nutrient materials but play a role as regulatory (signaling) molecules.

One of the unusual features of BCAAs is that they are primarily catabolized in muscles, whereas other essential amino acids are mainly catabolized in the liver. Half of the total activity of BCAA catabolic enzymes (branched-chain amino acid transferase (BCAT) and branched-chain keto acid dehydrogenase (BCKD)) is observed in the skeletal muscle [35]. BCAA catabolism is promoted by exercise.

The increase in circulating BCAA levels after a protein-containing meal is "sensed" by a number of different tissues, and it has important effects in these particular tissues. Thus, BCAAs serve as important signals to other tissues. A protein kinase, known as mTOR (mammalian target of rapamycin), is the intracellular target during stimulation of protein synthesis [36]. It is also the target for insulin, but the precise mechanism is still to be revealed.

BCAAs also function to suppress the production of lactic acid, a fatigue-causing substance. With continued exercise, lactic acid levels in the blood increase. Thus, the pH in the muscle decreases, leading to difficulty in muscle contraction and subsequent muscle fatigue. However, replenishment of BCAAs inhibits the elevation of lactic acid levels in the blood and prevents muscle fatigue.

3.3 Branched-Chain Amino Acids for the Elderly

Sarcopenia is the degenerative loss of skeletal muscle mass, quality, and strength associated with aging. It is considered a major issue related to the aging population. In some countries such as Japan, average life spans are increasing, and the overall population is aging. Patients with sarcopenia lose muscle mass because of imbalanced protein synthesis, and the muscle is broken down as they age.

The leucine-enriched essential amino acid supplementation, particularly coupled with exercise, effectively enhanced the muscle mass, muscle strength, and walking speed in women with sarcopenia [37]. In this study, four groups of 39 women, over 75 years, were monitored to observe the effect of amino acid intake and exercise on muscle growth and strength (Fig. 4). Amino acid intake remarkably improved the effectiveness of workouts, and physical exercise in turn increased the muscle mass and strength. The results show that amino acid intake could reverse the loss in muscle mass and strength observed with aging. BCAA supplementation is highly encouraged for allowing people to lead healthier lives in an aging society.

One study evaluated the effect of high-leucine intake on muscle protein metabolism in the elderly and young individuals. They revealed that increasing the

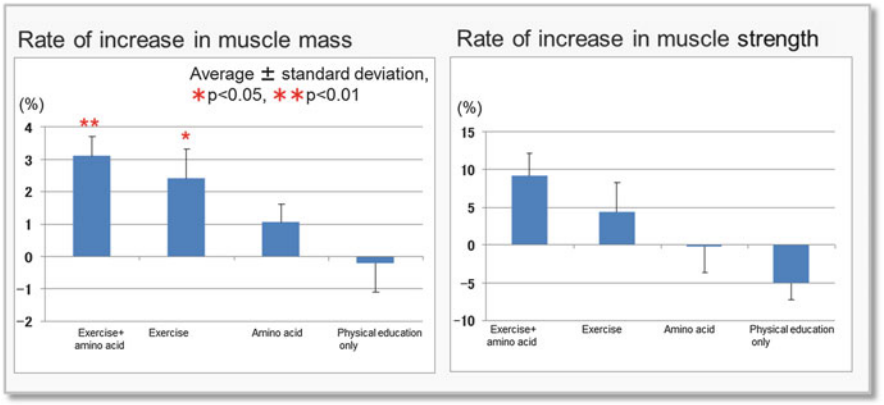

Fig. 4 BCAAs prevent the degradation of motor skills in the elderly. Methodology: The subjects were women with declining muscle mass/muscle strength (ages over 75 years). Each subjects' amino acid intake was 3 g twice a day. Each exercise program lasted for 60 min and was repeated twice a week for 3 months. Modified from [37]

proportion of leucine intake can reverse an attenuated response of muscle protein synthesis in the elderly, but it does not result in further stimulation of muscle protein synthesis in young individuals [38].

Furthermore, ingestion of extra leucine may be particularly important for the stimulation of skeletal muscle protein synthesis.

3.4 Therapeutic Nutrients for Hepatic Failure

BCAAs constitute approximately 40% of the free essential amino acids in the blood plasma, and they are used as an energy source. In patients with liver cirrhosis, a significant decrease in BCAA levels in the plasma may lead to malnutrition or severe hepatic encephalopathy. It was found that BCAA supplementation to such patients improved the nutritional status and lengthened patient survival time.

4 Glycine: For a Good Night's Sleep

Glycine, a nonessential amino acid, is synthesized endogenously and plays an essential role in the peripheral and central nervous systems. It had been reported that orally administered glycine shows beneficial effects on memory and attention in health volunteers, without the pharmacological effect on subjective mood observed during administration of a central nervous system (CNS) stimulant [39].

Recently, it was found that glycine ingestion before bedtime significantly improved subjective sleep quality in human volunteers who had been continuously experiencing unsatisfactory sleep.

Discovery of the effect began with a preliminary observation in healthy volunteers, who ingested placebo and experienced an improvement in sleep quality. Glycine is often considered a biologically neutral molecule, and it is used as a placebo control in amino acid supplementation studies. The effect was examined and confirmed in a randomized, double-blinded, crossover trial [40].

The effects of glycine on subjective sleep quality were assessed using the St Mary's Hospital (SMH) Sleep Questionnaire [41]. For questions such as Q11 "How satisfied were you with last night's sleep?" significant beneficial effects of glycine were revealed. This result meant that these individuals were more satisfied with their sleep.

Possible mechanisms for this effect of glycine were investigated [42]. Oral administration of glycine to rats was found to induce a significant decrease in the core body temperature (CBT) associated with an increase in cutaneous blood flow. The onset of sleep is known to involve a decrease in the CBT.

A "good night's sleep" is no doubt important for most people. It was reported that approximately 30% of the general population suffer from symptoms of insomnia [43]. The use of hypnotics, such as benzodiazepines, is widespread. However,

these hypnotics alter sleep architecture and have well-known problems such as the induction of daytime sleepiness and a reduction of daytime cognitive function. Glycine's mode of action is unique and very different from that of hypnotics. Glycine does not modify the sleep architecture itself. It is a safe and reliable sleep regulator for occasions when sleep is disturbed. It improves sleep quality, leading to a natural sleep pattern.

5 Alanine and Glutamine: Acceleration of Alcohol Metabolism

It has been reported that rats fed an ethanol-containing diet tend to select alanine or glutamine and that intake of the alanine–glycine (ala–gln) combination accelerates the metabolism of ethanol in rats [44]. It is supposed that this effect is caused by the consumption of NADH that is generated during the oxidation of ethanol through glycogenesis from the ala–gln combination. The effect of alanine and glutamine supplementation on human ethanol metabolism has been evaluated [45].

This test was performed as follows: after intake of alcohol (white wine), 22 healthy adult males were fed a diet containing ala–gln or a placebo (sugar). The amount of alcohol in the breath was measured. The group fed the ala–gln diet showed quicker degradation of alcohol (Fig. 5). It was concluded that the combination of alanine and glutamine accelerates the metabolism of alcohol in the liver.

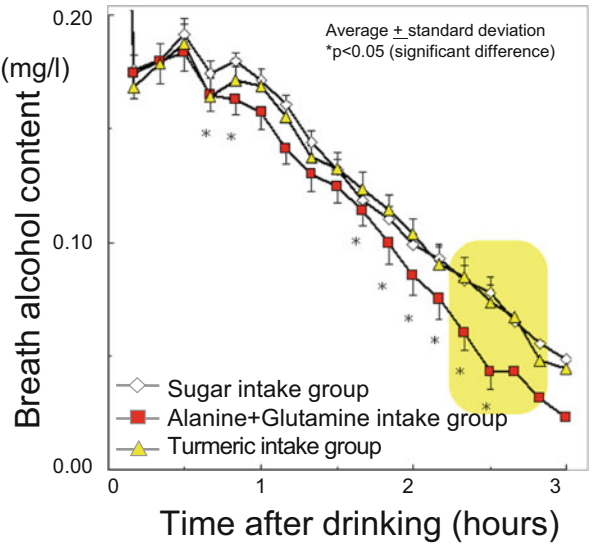

Fig. 5 Acceleration of alcohol metabolism by alanine and glutamine. This test was performed after intake of alcohol (white wine); 22 healthy adult males consumed diets containing alanine–glutamine or placebo (sugar). Then, the amount of alcohol in the breath was measured. It was observed that ingesting alanine and glutamine results in quicker degradation of alcohol. Alanine and glutamine accelerate the processing of alcohol in the liver. Modified from [45]

6 Cystine and Theanine: Prevention of Cold

Cystine is a precursor of glutathione (GSH), which is responsible for antioxidant activity in the body. The supply of cystine is the rate-limiting step of GSH synthesis. On the other hand, theanine (γ-glutamylethylamide) is contained in green tea and is known to be metabolized in the gut or liver into glutamate and ethylamine. It had been reported that the addition of cystine and theanine leads to increased amounts of GSH in macrophages [46].

In the experiments in mice, oral administration of L-cystine and L-theanine (CT) reinforced antigen-specific antibody production after antigen stimulation; these effects may have been caused by the reinforcement of glutathione (GSH) synthesis and the humoral immune response [47, 48]. It was suggested from these results that cystine and theanine may improve immunity.

Then, a randomized, placebo-controlled, 5-week trial with 176 volunteers was carried out to evaluate the effects of CT against common cold in humans (Fig. 6, [49]). The results indicated that CT supplementation significantly reduced the incidence of colds. The percentage of subjects who caught colds was lower among those who ingested CT. These results suggest that CT supplementation may be useful for the prevention of the common cold.

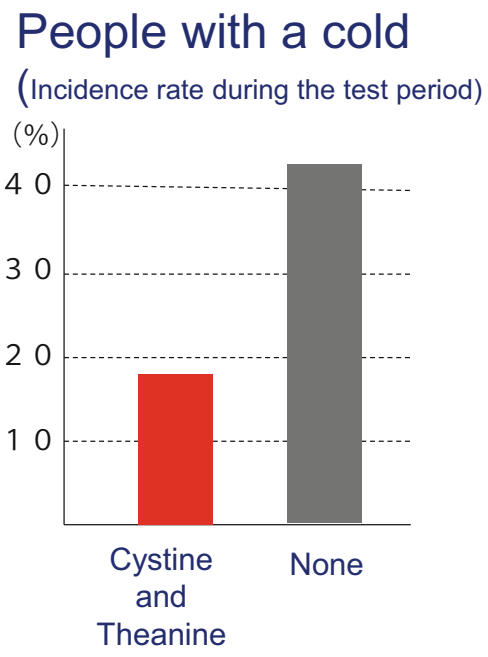

Fig. 6 Effect of L-cystine and L-theanine to prevent colds. The percentage of people with a cold in winter was measured. Intake of cystine and theanine resulted in a decreased rate. Cystine and theanine may improve immunity

7 Application as a Diagnostic Indicator for Cancers

Finally, amino acids are used as indicators in new clinical diagnostic methods.

The free amino acid composition of human blood plasma is constant. However, it was recently found that the composition changes when one contracts a disease, such as various cancers and diabetes. It was also found that the pattern of free amino acid composition changes depending on the type of cancer. Since the pattern of the change in free amino acid composition changes with differences in the kind of cancer, the pattern of change is useful for the evaluation of the risk of various cancers.

Therefore, the risk of various cancers can be estimated by measuring the free amino acid composition of blood plasma. Most of the present diagnostic tools assess the risk of only one type of cancer or damage the body. But in this method, the risks of several types of cancers are assessed by measuring only the amino acid composition in a small portion of a blood sample. This method was established in 2011 and is now receiving much attention as a robust tool for health assessment.

8 Conclusion

Amino acids has contributed to resolve issues in various fields such as food, infusions, clinical diets, pharmaceuticals, cosmetics, feed, and fertilizer. However, there still remain a lot of issues for humans, such as malnutrition, obesity, cancers, aging, and stress, as well as pollution of the air, water, and soil. The functions of amino acids being discovered must realistically contribute to the resolution of issues.

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Toward Sustainable Amino Acid Production

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Abstract Because the global amino acid production industry has been growing steadily and is expected to grow even more in the future, efficient production by fermentation is of great importance from economic and sustainability viewpoints. Many systems biology technologies, such as genome breeding, omics analysis, metabolic flux analysis, and metabolic simulation, have been employed for the improvement of amino acid-producing strains of bacteria. Synthetic biological approaches have recently been applied to strain development. It is also important to use sustainable carbon sources, such as glycerol or pyrolytic sugars from cellulosic biomass, instead of conventional carbon sources, such as glucose or sucrose, which can be used as food. Furthermore, reduction of sub-raw substrates has been shown to lead to reduction of environmental burdens and cost. Recently, a new fermentation system for glutamate production under acidic pH was developed to decrease the amount of one sub-raw material, ammonium, for maintenance of culture pH. At the same time, the utilization of fermentation coproducts, such as cells, ammonium sulfate, and fermentation broth, is a useful approach to decrease waste. In this chapter, further perspectives for future amino acid fermentation from one-carbon compounds are described.

Keywords Amino acid, Coproduct, Fermentation, One-carbon compound, Raw material, Sub-raw material, Synthetic biology, Systems biology

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Contents

1 Systems Biology and Synthetic Biological Approaches

Since the genome sequence of the model and industrial organism *Escherichia coli* has been determined by Blattner et al. [1], the genome sequences of *Corynebacterium glutamicum* ATCC 13032 [2, 3]; *C. efficiens*, a near relative of *C. glutamicum* with different optimal growth temperatures [4]; *C. glutamicum* strain R [5]; *C. glutamicum* ATCC 14067 [formerly *Brevibacterium flavum*] [6]; and *C. glutamicum* AJ 1511 [formerly *Brevibacterium lactofermentum*] [7] have been determined. On the basis of genomic information, systems biology approaches have been applied to maximize the efficiency of amino acid production [8]. Genome breeding consisting of characterization and reconstitution of a mutation set essential for high-level production of amino acids was proposed by identifying mutations in the L-lysine-producer of *C. glutamicum* [9]. Omics analyses, such as transcriptomic, proteomic, and metabolomic analyses, have been extensively applied for improvement of amino acid producers [10, 11].

Metabolic flux analysis (MFA) using ^{13}C labeling is a powerful method for quantifying intracellular reaction rates within a metabolic network. In this approach, intracellular flux is calculated using a stoichiometric model for the major intracellular reactions and applying mass balances around intracellular metabolites using a set of measured extracellular fluxes, typically uptake rates of substrates and secretion rates of metabolites, as input for the calculations [12]. Since the first report on analysis of L-lysine production [13], MFA has been used for elucidating the in vivo metabolic state of cells during fermentation [14]. Recently, the development of MFA and its application to improvement of *C. glutamicum* amino acid producers has been reviewed in detail [15]. Integration of MFA data from different mutants and/or different cultural environments with different levels of omics analysis information using a systems biology approach is expected to reveal the roles of global regulators, which are quite important for metabolic regulation during amino acid fermentation [16]. Genome-scale metabolic models for *E. coli* [17] and *C. glutamicum* [18] have been developed and widely

used for estimation of, among other parameters, growth capability and amino acid production, using flux balance analysis.

Kinetic modeling is also valuable to industrial biotechnology and is able to assist in the rational design of cell factory properties or production processes in which they are utilized because kinetic models are capable of representing the complex biochemistry of cells more completely than most other types of models. However, several challenges must be overcome before kinetic modeling can reach the degree of maturity required for routine application in industry [19]. One of the targets of kinetic modeling has been the phosphotransferase system of *E. coli*, which is quite important for material production because it defines substrate uptake rates [20, 21]. Large-scale kinetic modeling and dynamic simulation, including the phosphotransferase system, glycolysis, and the pentose phosphate pathway but excluding the regulatory network of *E. coli*, has been reported [22]. Dynamic simulation of glutamate fermentation with large-scale kinetic modeling, including the central metabolic pathway regulatory network, has been reported [23], and extensive sensitivity analysis and validation have been performed [24].

Recently, synthetic biological approaches have been applied to strain development [25]. A successful example wherein a new pathway was introduced to improve amino acid yield involves phosphoketolase (PKT). PKT catalyzes the following reactions:

$$\text{Fructose 6-phosphate} + P_i \rightarrow \text{Acetyl phosphate} + \text{Erythrose 4-phosphate} \quad (1)$$

$$\text{Xylulose 5-phosphate} + P_i \rightarrow \text{Acetyl phosphate} + \text{Glyceraldehyde 3-phosphate} \quad (2)$$

PKT can increase the maximum theoretical yield of L-glutamate from glucose up to 98.0% by weight (120% mol/mol L-glutamate produced/glucose consumed) by bypassing the CO_2-releasing pyruvate dehydrogenase reaction (Fig. 1). The *xfp* gene encoding PKT was cloned from *Bifidobacterium animalis* and overexpressed under a strong *cspB* promoter in the L-glutamate-producing strain of *C. glutamicum* (Δ*odhA* mutant). When cells of this producer strain with and without the *xfp* gene were cultivated in a controlled fermentation system, L-glutamate production from the *xfp*-expressing strain was much higher than that of the original strain coupled with suppressed CO_2 emission [26]. Recently, nonoxidative glycolysis has been suggested in which the PKT pathway enables complete carbon conservation in sugar catabolism to acetyl coenzyme A [27]. The enzyme mixture, PKT from *Bifidobacterium adolescentis*, transaldolase, transketolase, fructose 1,6-bisphosphate, ribulose 5-phosphate epimerase, ribose 5-phosphate isomerase, fructose 1,6-bisphosphate aldolase, and triose phosphate isomerase and ATP have been shown to convert one fructose 6-phosphate molecule to three acetyl phosphate molecules in vitro. Xylose was converted to acetate and other products under anaerobic conditions. The JCL118 strain (Δ*ldhA* Δ*adhE* Δ*frdBC* Δ*pflB*), which overexpresses PKT from *Bifidobacterium adolescentis* and fructose 1,6-bisphosphate from *E. coli*, produced acetate from xylose with a near theoretical

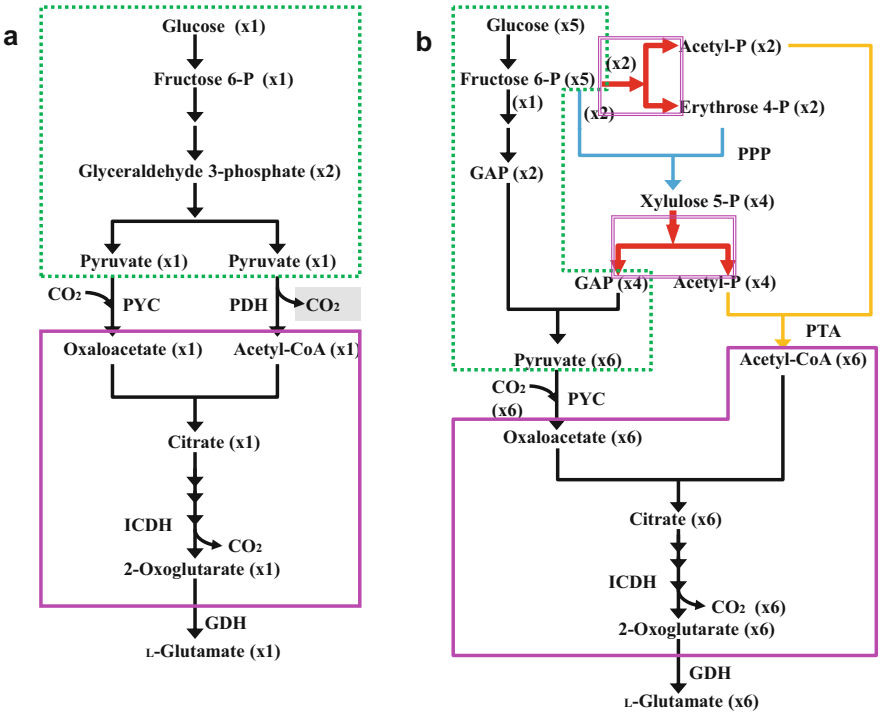

Fig. 1 The phosphoketolase (PKT) pathway. Biosynthesis of L-glutamate from glucose (modified from Chinen et al. [26]). Glycolysis and the oxidative branch of the tricarboxylic acid cycle are enclosed by *dotted line* and *solid line*, respectively. The number of compounds involved in each metabolic reaction under ideal conditions is shown in *parentheses*. (**a**) Metabolic pathway of wild-type *Corynebacterium glutamicum* showing maximum theoretical yield of L-glutamate from glucose. Emission of CO_2 induced by pyruvate dehydrogenase (PDH) is indicated by the *panel*. (**b**) Metabolic pathway engineered by introduction of phosphoketolase (indicated and enclosed by *double line*) to bypass CO_2 emission. *PPP* pentose phosphate pathway, *PTA* phosphotransacetylase. This pathway produces 6 mol of L-glutamate from 5 mol of glucose without the loss of carbon via CO_2 release

ratio of acetate/xylose in vivo [27]. Utilization of the PKT pathway enables complete carbon conservation in sugar catabolism to acetyl coenzyme A and is expected to be used in conjunction with CO_2 fixation and other one-carbon (C1) assimilation pathways to achieve a 100% carbon yield from various substances.

2 Alternative Raw Materials

Currently, most amino acids are produced mainly from sugars, such as glucose or sucrose, which are also used as foods. From a sustainability viewpoint, raw materials used for amino acid fermentation should avoid competition with food resources. The candidates for nonfood raw materials are glycerol, a valuable by-product in biodiesel production, and glucose and C5 sugars from cellulosic biomass by enzymatic hydrolysis.

Glycerol, also known as glycerin, is a by-product of a transesterification reaction used in biodiesel factories. Glycerol produced from biodiesel factories is crude and contains various impurities. Crude glycerol obtained from biodiesel factories consists of glycerol, water, organic and inorganic salts, soap, alcohol, and traces of glycerides. However, crude glycerol could be used as an organic carbon substrate for the production of value-added chemicals, such as 1,3-propanediol, organic acids, or polyols, by microorganisms [28].

C. glutamicum, which cannot utilize glycerol naturally, was engineered for glycerol utilization by heterologous expression of *E. coli* aerobic glycerol utilization genes encoding a glycerol facilitator (*glpF*), glycerol kinase (*glpK*), and glycerol-3-phosphate dehydrogenase (*glpD*). *C. glutamicum* strains expressing these genes show fast growth with glycerol as the sole carbon source and similar L-glutamate and L-lysine production properties to those of glucose [29]. Meiswinkel et al. [30] reported growth of recombinant strains expressing *glpF*, *glpK*, and *glpD* from *E. coli* fed crude glycerol from biodiesel factories. Besides growth, production of the amino acids L-glutamate, L-lysine, and L-arginine was shown to be dependent on the quality of crude glycerol from biodiesel factories [30]. Because *E. coli* has been known to utilize glycerol, many amino acids produced by *E. coli* can also be produced from glycerol.

Lignocellulosic materials containing cellulose, hemicellulose, and lignin are the most abundant renewable organic resource on earth. Conversion of both cellulose (glucose) and hemicellulose (hexose and pentose) in the production of ethanol has been studied intensively with the aim of developing a technically and economically viable bioprocess for fuel production. Thus, utilization of lignocellulosic materials as a renewable resource for energy and various chemicals is expected to increase.

Xylose and arabinose are the major pentose constituents in hemicellulose. However, wild-type *C. glutamicum* strains cannot utilize the pentose fractions of lignocellulosic hydrolysates. Previously, introduction of the xylose operon from *E. coli* was shown to enable growth of *C. glutamicum* on xylose [31], and implementation of the *E. coli araABD* gene cluster enabled growth and production of amino acids on this carbon source [32]. Meiswinkel et al. [33] found that introduction of the *Xanthomonas campestris* xylose isomerase gene and *C. glutamicum* xylulokinase gene doubled the growth rate and increased glutamate productivity of *C. glutamicum* from that of the strain solely expressing the *E. coli* xylose isomerase gene. Furthermore, Gopinath et al. [34] reported that recombinant pentose-utilizing

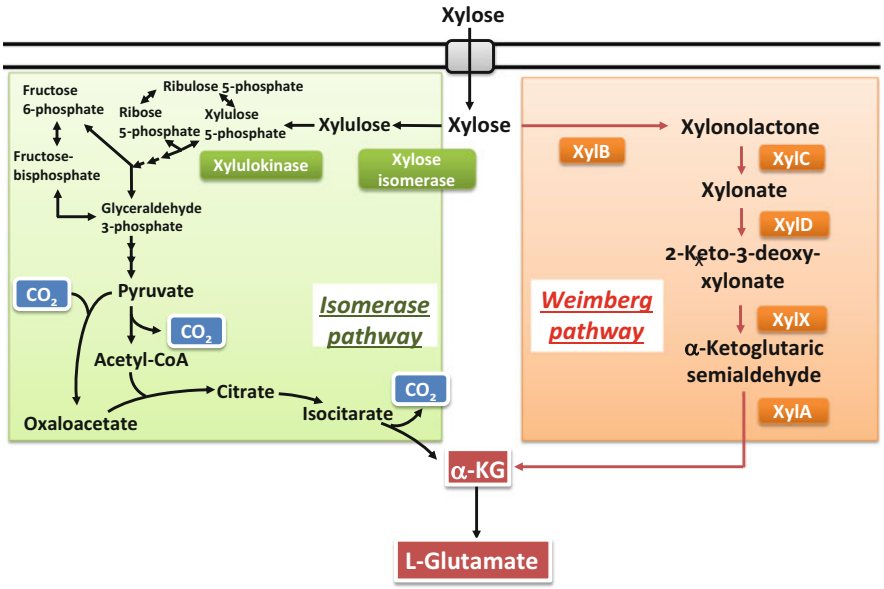

Fig. 2 Xylose utilization pathways. Biosynthesis of L-glutamate from xylose. The isomerase pathway is accompanied by carbon loss (*left*). The Weimberg pathway encoded by the *xylXABCD* operon produces 1 mol of L-glutamate from 1 mol of xylose without the loss of carbon via CO_2 release

strains derived from an L-lysine-producing *C. glutamicum* strain utilized arabinose and/or xylose, which were present in acid hydrolysates of rice straw or wheat bran.

In contrast to the commonly known isomerase pathway that leads to significant carbon loss in the form of CO_2, the Weimberg pathway (Fig. 2), first discovered in *Pseudomonas fragi* and later in *Haloferax volcanii* and *Caulobacter crescentus* [35–37], is an attractive alternative for xylose assimilation. In this five-step oxidative pathway, xylose is exclusively oxidized to the C5 compound α-ketoglutarate without carbon loss (Fig. 2). Previously, the Weimberg pathway encoded by the *xylXABCD* operon from *Caulobacter crescentus* was introduced into *C. glutamicum* and enabled a recombinant *C. glutamicum* strain to utilize xylose where it is the sole carbon source and in xylose/glucose mixtures [38]. The recombinant strain efficiently produced L-glutamate from xylose, and its L-glutamate productivity was higher than that of a strain carrying a gene encoding xylose isomerase by which *C. glutamicum* could assimilate xylose (Yamada et al. unpublished).

Direct utilization of cellulose or hemicellulose is one major goal. Adham et al. [39] expressed xylanase Xys1 and the cellulase Cel1 from the straw-decomposing *Streptomyces halstedii* JM8 in *C. glutamicum* ATCC 13869. Hyeon et al. [40] reported development of a *C. glutamicum* strain expressing functional minicellulosomes containing chimeric endoglucanase E consisting of the endoglucanase E catalytic backbone of *Clostridium thermocellum* fused with the endoglucanase B dockerin domain of *Clostridium cellulovorans*. The engineered

strain degraded carboxymethyl cellulose efficiently by substrate targeting via the carbohydrate-binding module [40]. Tsuchidate et al. [41] expressed endoglucanase from *Clostridium cellulovorans* 743B in *C. glutamicum* using the *E. coli torA* signal sequence. The secreted endoglucanase produced 123 mg of reducing sugar from 5 g of β-glucan after 72 h at 30°C. Moreover, L-glutamate fermentation from β-glucan with the addition of *Aspergillus aculeatus* β-glucosidase produced by recombinant *Aspergillus oryzae* resulted in 178 mg/L of L-glutamate from 15 g of β-glucan [41]. Recently, Kim et al. [42] reported that cellulase complexes containing two cellulolytic enzymes, endoglucanase E and β-glucosidase A from *Clostridium thermocellum*, anchored to the surface of *C. glutamicum* by a mechanosensitive channel synergistically lead to a 3.1- to 6.0-fold increase in the direct conversion of biomass (rice straw and *Miscanthus* and rape stem pretreated under high temperature with alkaline chemicals) to reducing sugars relative to conversion by secreted cellulase complexes.

Furthermore, fatty acids represent an alternative carbon source derived from biodiesel production or cooking oil wastes or produced by microalgae. Doi et al. [43] reported that fatty acids can be used as raw materials for L-lysine fermentation and that reduction of hydrogen peroxide stress derived from fatty acid β-oxidation improved fatty acid utilization in *E. coli*. Acetate and ethanol are also expected to be alternative carbon sources because *C. glutamicum* can grow on these substances [44, 45].

3 Reduction of Sub-raw Materials by a New Fermentation System

3.1 Concept of the New Fermentation System

Because L-glutamate is an acidic amino acid, the bacterial growth medium is acidified in accordance with L-glutamate accumulation during fermentation. Generally, L-glutamate fermentation in *C. glutamicum* is performed at a neutral pH. Therefore, addition of a large amount of alkali, usually ammonia, is necessary to maintain the pH of the medium. L-Glutamate in the culture medium is stored as ammonium salt. After fermentation, L-glutamate is crystallized by addition of acid, usually sulfuric acid or hydrochloric acid, utilizing the low solubility of this amino acid in acidic conditions (Fig. 3). The final product, monosodium glutamate, is produced by neutralizing L-glutamate with sodium hydroxide. In this traditional manufacturing process, large amounts of alkali and acid are used in the fermentation and crystal isolation steps, respectively, and a large amount of by-product salt, such as ammonium sulfate, is produced.

In recent years, production of L-glutamate by integrating fermentation and isolation steps has been called "L-glutamate crystallization fermentation." In this novel method, crystallization of L-glutamate occurs during the fermentation

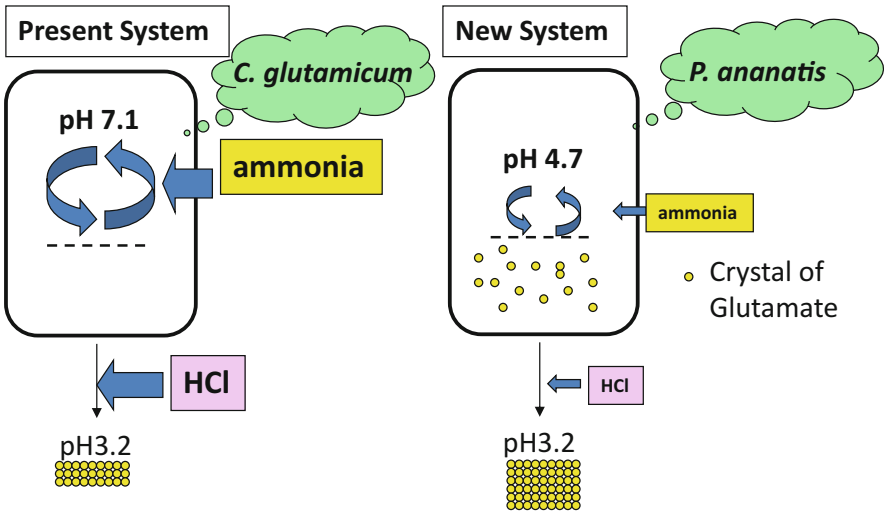

Fig. 3 New fermentation system for reduction of sub-raw materials. Fermentation under acidic conditions leads a decreased input of ammonia and hydrochloric acid. In addition, because the solubility of glutamate is low in acidic conditions, a large amount of glutamate accumulates as crystals in the fermentation tank

process. L-Glutamate has two carboxyl groups and one amino group, and its theoretical isoelectric point is pH 3.22. Due to its acidity, the solubility of L-glutamate in acidic conditions is low. If L-glutamate fermentation is performed under acidic conditions, a large portion of the L-glutamate would lose its electrical charge and precipitate as crystals. Thus, much of the counter ion ammonia, as well as the acid added and salt produced in the purification step, would be reduced. For example, when fermentation is performed at pH 4.5 and 7, the amount of ammonia and acid needed for fermentation and crystallization would be decreased by 40% and 80%, respectively.

3.2 *Isolation of the Host Strain*

Because L-glutamate crystallization fermentation requires acidic fermentation conditions, it is essential that the host be able to grow at a low pH and resist high concentrations of glutamate. After screening various strains for these properties, *Pantoea ananatis* strain AJ13355 isolated from the soil of a tea plantation was selected. In the current study, the complete genomic sequence of *P. ananatis* AJ13355 was determined and found to consist of a single, circular chromosome consisting of 4,555,536 bp (DDBJ: AP012032) and a circular plasmid (pEA320) with 321,744 bp (DDBJ: AP012033). After automated annotation, 4,071 protein-coding sequences were identified in the *P. ananatis* AJ13355 genome [46].

3.3 Construction of Glutamate-Producing Bacteria

Although *P. ananatis* AJ13355 possesses all of the genes needed for biosynthesis of glutamate, L-glutamate accumulation was not induced by penicillin or surfactants, unlike in *C. glutamicum*. Therefore, a strain which overproduces glutamate by modifying the L-glutamate biosynthetic pathway in *P. ananatis* AJ13355 was bred. To do so, enhancement of the biosynthetic pathway and knock out of degradation pathway genes of the target substance were used. Then, the L-glutamate producer was constructed through the following steps: (1) decreased extracellular polysaccharide production, (2) weakened 2-oxoglutarate dehydrogenase complex activity, and (3) enhanced activity of key enzymes in glutamate biosynthesis, such as citrate synthase, phosphoenolpyruvate carboxylase, and glutamate dehydrogenase. However, glutamate production deteriorated, and accumulation of by-products, such as acetoin and 2,3-butanediol, was observed, especially when cultured at an acidic pH. L-Glutamate loses its electrical charge under acidic conditions and can easily pass through the hydrophobic cell membrane. When the cellular pH was maintained at near 7, L-glutamate flowed into the cell and reionized, making it unable to cross the membrane again. Consequently, L-glutamate was concentrated inside the cell, which led to feedback inhibition of L-glutamate biosynthesis.

There are two types of citrate synthase enzymes. Type I enzymes are found in *C. glutamicum* and other Gram-positive bacteria, and type II enzymes are found in Gram-negative bacteria. Type II enzyme activity is inhibited by NADH allosterically and α-ketoglutarate. In a recent study, type II citrate synthases were found to be inhibited by high concentrations of glutamate, while type I enzymes were not, and introduction of type I citrate synthase from *C. glutamicum* effectively increased glutamate production.

3.4 Glutamate Production with Crystallization

L-glutamate production was improved by enhancement of type I citrate synthase activity. However, an extremely high concentration of L-glutamate in the medium still had a negative effect on cell growth and sugar consumption. Therefore, a mutant *P. ananatis* AJ13601 strain with glutamate resistance was isolated. This mutant strain produced 82 g/L of glutamate at pH 4.5 with glucose as the carbon source. Because the solubility of glutamate at that pH is 41 g/L, a large amount accumulated as crystals. This is the first example of L-glutamate crystallization fermentation. L-glutamate crystallization fermentation not only decreases the cost of acid and alkali but also the burden on the environment. Thus, this new process is a promising method of sustainable amino acid production.

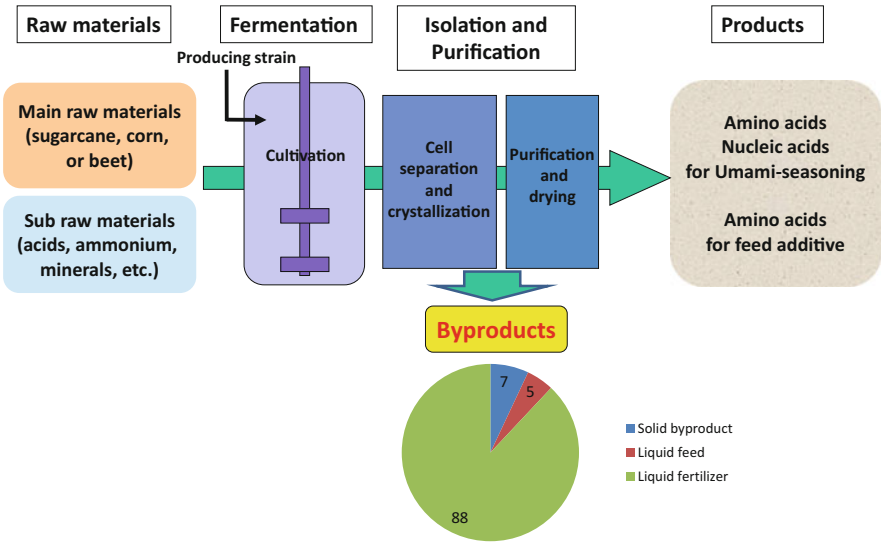

Fig. 4 Utilization of amino acid fermentation coproducts. Amino acid by-products are generated during the purification and isolation process. Coproducts are typically distributed as solid by-products (7%), liquid feeds (5%), and liquid fertilizers (88%)

4 Utilization of Coproducts

Amino acid manufacturing processes generate waste, atmospheric emissions, and drainage water. To minimize the environmental impact and ensure production sustainability, utilization of by-products of amino acid fermentation is critically important. These by-products are nutrient-rich and used as coproducts in agricultural, animal, and aquatic products industries around the world. The examples sold by Ajinomoto Co., Inc., described below were also shown in the Ajinomoto Group Sustainability Report 2012 [47]. A schematic representation of by-product formation in the amino acid production process, as well as the ratio of solid (7%) and liquid (93%) content in coproducts, is shown in Fig. 4. By-products are generated during purification and isolation after fermentation. Approximately 90% of the by-products are transformed into coproducts which are mainly used in slow-release solid fertilizer, foliar fertilizer, dried bacterial cells, and silage modifying agents.

Solid by-products contain salts, waste activated carbon, excess sludge, and waste filter aids. These can be used in, for example, animal and fish feeds, raw material for solid fertilizers, soil conditioners, raw material for cement, and fuels. AJIFOL® foliar fertilizer enables plants to effectively absorb nutrients through their leaves and is a prime example of a value-added coproduct. AJIFOL® liquid fertilizers are produced from the fermentation of L-glutamate and contain macro- and micronutrients, nitrogen and potassium, as well as several additional amino acids. These fertilizers were first launched in 1988 in Brazil and have since been used in many countries, including Brazil, Peru, the United States, Thailand, Vietnam,

Indonesia, and Japan. The benefits of AJIFOL® include (1) increased plant growth and development, as well as improved crop production and quality, especially under stressful conditions, by effectively providing macro- and micronutrients required by plants, and (2) increased resistance to diseases due to the elicitor activity of amino acids.

In Japan, the liquid fertilizer AMIHEART®, derived from the fermentation of inosine using natto bacteria (*Bacillus amyloliquefaciens*), was launched in 2011. This fertilizer is rich in inosine and has been proven effective in fostering the rooting of plants, promoting rapid growth, and increasing crop yields. It is mainly used for melons, strawberries, tomatoes, bell peppers, eggplant, and tea cultivation and is now beginning to be used in rice nursery production.

AJITEIN®, high value-added protein feeds containing bacterial cells by using protein-rich bacterial cells separated from coproducts that are often used as liquid fertilizers and feeds (Fig. 3), is distributed throughout Indonesia as an alternative to fish and soybean meal. One of the advantages that AJITEIN® possesses over the other protein sources is that it contains β-glucan, which can stimulate and enhance the immune system of livestock. The liquid fertilizers AMI-AMI® and AMINAR® are representative coproducts of Ajinomoto group companies in Thailand, Vietnam, and Indonesia. These fertilizers are mainly utilized for cultivation of major crops, such as rice, corn, soybeans, and sugar cane, as well as vegetables and fruits. They derive from the liquid coproducts of monosodium glutamate production, which are rich in protein and free amino acids. Thus, they can be applied as an alternative source of protein in the manufacture of animal and fish feed.

5 Toward Further Sustainable Amino Acid Fermentation

For more than 50 years, the fermentation of amino acids has continuously improved. However, most amino acids are still mainly produced from sugars, such as glucose or sucrose, which are food raw materials. The candidates for nonfood raw materials are glycerol, a valuable by-product in biodiesel production by transesterification, as well as glucose and C5 sugars from cellulosic biomass (Sect. 2).

C1 compounds occur abundantly in nature. Methane and methanol are two of the most important C1 compounds from a biotechnological and bulk chemical viewpoint. The possibility of utilizing the C1 substrates methane and methanol as alternative nonfood feedstocks has attracted high scientific interest. This interest is largely based on economic considerations (i.e., low methanol costs and increased capacity for methanol production worldwide) and technological advances, which allow for production of important industrial chemicals from methanol as a feedstock using microbes. Generally, methanol is prepared by reaction of a mixture of carbon oxides (CO and CO_2) with hydrogen; the CO/CO_2 mixture, in turn, derives from methane generally obtained from natural gas (e.g., shale gas). Methanol is being increasingly produced from renewable sources. The biomass is gasified, and

the resulting gas, a mixture of CO, H_2, and CO_2, satisfies the quality requirements of methanol synthesis. In the long term, more renewable/bio-based methanol use in, for example, shipping and transportation fuels is envisioned. However, methane and methanol utilization has not yet been commercialized because the price of methanol still makes it a more expensive substrate than sugar for bulk amino acid production.

Methylotrophs comprise a large number of both aerobic and anaerobic microorganisms that can grow in methane and methanol. Obligate methylotrophs can exclusively utilize C1 compounds as a sole carbon and energy source, while facultative methylotrophs can utilize both C1 and multicarbon compounds. Genetic tools for many methylotrophs have been established, and engineering of methylotrophs leading to overproduction of different amino acids has been reported [48]. For example, the Gram-negative obligate methylotroph *Methylophilus methylotrophus* synthesized 1 g/L of L-lysine at 37°C through expression of a mutant gene encoding dihydrodipicolinate synthase that is deregulated by L-lysine inhibition [49]. By coexpressing a mutant gene encoding an L-lysine transporter, LysE from recombinant *C. glutamicum* accumulated 11.3 g/L of L-lysine from methanol [50]. A recombinant mutant of the Gram-negative obligate methylotroph *Methylobacillus glycogenes* overexpressing a dihydrodipicolinate synthase that is partly desensitized to inhibition by L-lysine was reported to produce approximately 8 g/L of L-lysine and 37 g/L of L-glutamate from methanol at 37°C [51].

Bacillus methanolicus is a Gram-positive, facultative methylotrophic and thermophilic bacterium considered to be one of the few candidates with the potential to convert methanol to value-added products and amino acids at high temperatures [48, 52]. The *Bacillus methanolicus* classical mutant with homoserine auxotrophy and resistance to amino acid analogs derived from strain NOA2 has been reported to secrete 37 g/L of L-lysine in fed-batch bioreactors [53]. *Bacillus methanolicus* MGA3 (ATCC 53907) has been shown to secrete 55 g/L of L-glutamate at 50°C with methanol as a carbon source in fed-batch bioreactors [54]. Considering its ability to produce high concentrations of L-glutamate and L-lysine at 50°C, *Bacillus methanolicus* represents a promising microorganism for industrial-scale production processes.

As an alternative approach to engineering methylotrophs for production of amino acids from methanol, it may be possible to exploit the ability to utilize methanol as a carbon source in naturally nonmethylotrophic, amino acid-producing bacteria by introducing suitable heterologous pathways, such as the ribulose monophosphate or serine pathway. Witthoff et al. [55] showed the capability of *C. glutamicum* to oxidize methanol to CO_2 and identified the key enzymes involved in this endogenous pathway as a first step toward making *C. glutamicum* a methylotroph.

Direct conversion of CO_2 to amino acids is one of ultimate methods from an environmental viewpoint. Matsunaga et al. [56] reported the production of L-glutamate from CO_2 by the marine cyanobacterium *Synechococcus* sp. NKBG040607A using a biosolar reactor; the maximum CO_2-to-glutamate conversion ratio was 28% at a cell density of 3×10^8 cells/mL. L-glutamate

productivity using the biosolar reactor has been reported to be 15 μmol/L/h. Furthermore, Ryu et al. [57] reported a new type of artificial photosynthetic system that integrally and efficiently couples biocatalytic redox reactions with photocatalytic water splitting. Efficient coupling is achieved using tetracobalt polyoxometalate and a rhodium-based organometallic compound as hole and electron scavengers, respectively, for photoexcited $[Ru(bpy)_3]^{2+}$ that successively photosynthesize L-glutamate as a model compound using a model redox enzyme (L-glutamate dehydrogenase) upon in situ photoregeneration of cofactors. By unlocking new, beneficial amino acid functions, usage of amino acids has prevailed and is expected to expand in the future. Thus, efficient production of amino acids will continue to be explored further to help conserve and improve the global environment and maintain sustainable production.

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