Chapter 14
Microbial Metabolic Engineering for L-Threonine Production

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Abstract L-threonine, one of the three major amino acids produced throughout the world, has a wide application in industry, as an additive or as a precursor for the biosynthesis of other chemicals. It is predominantly produced through microbial fermentation the efficiency of which largely depends on the quality of strains. Metabolic engineering based on a cogent understanding of the metabolic pathways of L-threonine biosynthesis and regulation provides an effective alternative to the traditional breeding for strain development. Continuing efforts have been made in revealing the mechanisms and regulation of L-threonine producing strains, as well as in metabolic engineering of suitable organisms whereby genetically-defined, industrially competitive L-threonine producing strains have been successfully constructed. This review focuses on the global metabolic and regulatory networks responsible for L-threonine biosynthesis, the molecular mechanisms of regulation, and the strategies employed in strain engineering.

Keywords L-threonine biosynthesis • L-threonine production • Metabolic engineering • Regulation mechanism

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Abbreviations

SD sequence	Shine-Dalgarno sequence
ACT	Aspartate kinase, Chorismate mutase and TyrA

14.1 Introduction

L-threonine is currently one of the three major amino acids produced throughout the world with an annual market size of approximate 0.23 metric tons (Becker and Wittmann 2011). Among its wide industrial application, the most remarkable use of L-threonine is as a feed additive. Application of low protein level formula feeds supplemented with L-threonine improves the growth of livestock, relieves crude protein deficiency and lowers nitrogen emissions, thus contributing to the sustainable development of the society. Recent studies acknowledging L-threonine as the second and the third limiting amino acid in swine and poultry feeds respectively (Ajinomoto 2009) have stimulated the further expansion of the industry. Moreover, L-threonine can be used as precursor for the biosynthesis of L-isoleucine and L-homoalanine. This underpins recent developments in the efficient microbial production of L-threonine (Leuchtenberger et al. 2005).

The most efficient solution to improve productivity of the bioconversion and reduce costs is to develop highly productive strains. Thanks to traditional breeding methods, L-threonine producing strains of *Serratia marcescens* (Komatsubara et al. 1978), *Escherichia coli* (Furukawa et al. 1988) and *Corynebacterium glutamicum* (Morinage et al. 1987) have been developed. However, the traditional breeding procedures are time-consuming and labor-costly. Furthermore, strains surviving multiple rounds of random mutagenesis are genetically undefined and vulnerable to further changes, out of which at best either a marginal increase in yield or resistance to more stringent process requirements could be achieved. To overcome these difficulties in developing more efficient L-threonine producing strains, metabolic engineering through rational genetic manipulations offers a promising option for subsequent isolation of genetically defined hyper-producing strains. Selection criteria would include reduced by-product formation and expanded substrate spectra. Recruited strategies include increasing the biosynthetic metabolic flux of L-threonine, enhancing its excretion efficiency, and reducing unwanted carbon loss through the competing branches and its intracellular consumption. Most recently, systems metabolic engineering comprehensively combining all of these strategy elements has achieved success in restructuring a wild-type strain into a genetically definite, highly competitive L-threonine producer.

This chapter will focus on the global metabolic pathway of L-threonine together with regulation mechanisms and the progress in metabolic engineering of the dominating industrial bacteria, *E. coli* and *C. glutamicum*.

14.2 The Metabolic Pathway of L-Threonine Biosynthesis

L-threonine belongs to the aspartic family of amino acids. L-aspartate is synthesized from oxaloacetate, an intermediate of TCA cycle, by aspartate transaminase (Table 14.1) that is encoded by the *aspC* gene in *E. coli* (Fotheringham et al. 1986) and by *aspA* in *C. glutamicum* (Marienhagen et al. 2005). On substrates of carbohydrates,

Table 14.1 Enzymes and their coding genes related to the biosynthesis of L-threonine

Enzymes	Genes	Function
Phosphoenolpyruvate carboxylase	*ppc*	Carboxylate phosphoenolpyruvate to form oxaloacetate
Pyruvate carboxylase	*pyc*	Carboxylate pyruvate to form oxaloacetate
Aspartate transaminase	*aspC*; *aspA*	Transfer an amino group to oxaloacetate to form L-aspartate. The coding gene is *aspC* in *E. coli*, and is *aspA* in *C. glutamicum*
Aspartate kinase	*thrA*; *metL*; *lysC*	Phosphorylate aspartate to form aspartyl-P. There are three coding genes (*thrA*; *metL*; *lysC*) in *E. coli*, but only one (*lysC*) in *C. glutamicum*
Aspartyl semialdehyde dehydrogenase	*asd*	Deoxidize aspartyl-P to form aspartyl semialdehyde
Homoserine dehydrogenase	*thrA*; *metL*; *hom*	Remove the carboxyl group of aspartyl semialdehyde. There are two coding genes (*thrA*; *metL*) in *E. coli*, but only one (*hom*) in *C. glutamicum*
Homoserine kinase	*thrB*	Phosphorylate homoserine to form homoserine-P
Threonine synthase	*thrC*	Remove the phosphate group of homoserine-P to form threonine
Phosphoenolpyruvate carboxykinase	*pck*	Decarboxylate oxaloacetate to form phosphoenolpyruvate
Oxaloacetate decarboxylase	*odx*	Decarboxylate oxaloacetate to form pyruvate
Malic enzyme	*sfcA*; *maeB*; *malE*	Decarboxylate malate to form pyruvate. There are two coding genes (*sfcA*; *maeB*) in *E. coli*, but only one (*malE*) in *C. glutamicum*
Dihydrodipicolinate synthase	*dapA*	Consume L-aspartyl semialdehyde to produce L-lysine
Homoserine succinyltransferase	*metA*	Consume L-homoserine to produce L-methionine in *E. coli*
Homoserine acetyltransferase	*metX*	Consume L-homoserine to produce L-methionine in *E. coli*
Threonine dehydratase	*tdcB*; *ilvA*	Consume threonine to produce isoleucine
Threonine dehydrogenase	*tdh*	Consume threonine to produce glycine in *E. coli*
Threonine aldolase	*ltaE*	Consume threonine to produce glycine in *E. coli*
Serine hydroxymethyl transferase	*glyA*	Consume threonine to produce glycine in *C. glutamicum*
Permease	*rhtA*; *rhtB*; *rhtC*; *thrE*	Transport threonine from inside to the outside of cell. There are three coding genes (*rhtA*; *rhtB* and *rhtC*) in *E. coli*, but only one (*thrE*) in *C. glutamicum*

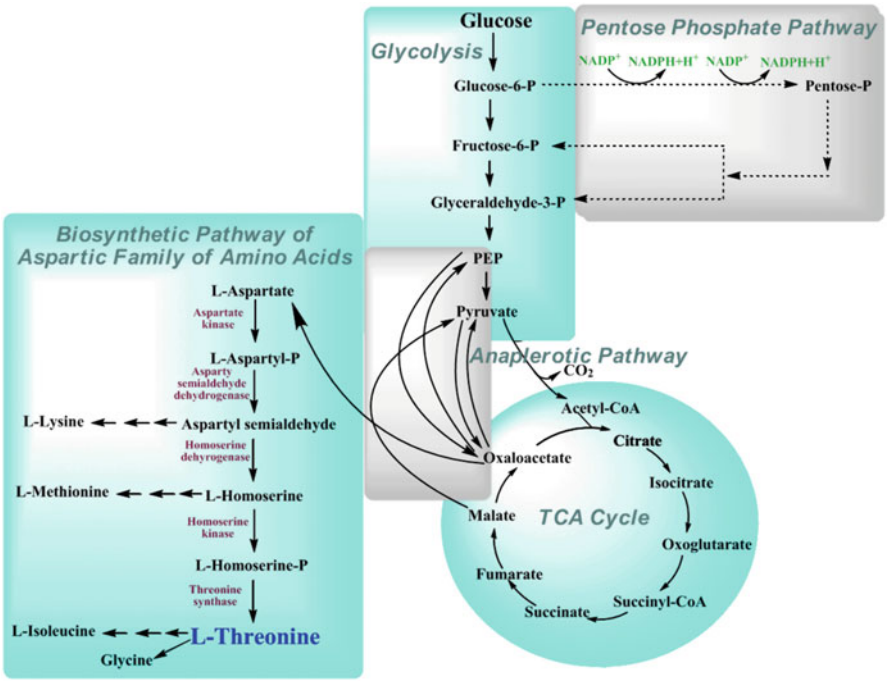

Fig. 14.1 The biosynthesis pathway of L-threonine. The pathway consists of centeral metabolic pathways and the threonine terminal pathways. The centeral metabolic pathways involve glycolysis, phosphate pentose pathway, TCA cycle and anaplerotic pathways. The threonine terminal pathway consists of five enzymetic steps. The first, third, and fourth reactions are catalyzed by the three key enzymes aspartate kinase, homoserine dehydrogenase, and homoserine kinase, respectively. There are four competing pathways that affect the biosynthesis of L-threonine, leading to formation of L-lysine, L-methionine, L-isoleucine, and glycine

L-threonine biosynthesis involves centeral metabolism including glycolysis, pentose phosphate pathway, TCA cycle and anaplerotic pathways between glycolysis and TCA cycle, prior to its terminal pathway (Fig. 14.1).

14.2.1 The Associated Anaplerotic Pathways

Anaplerotic reactions refer to C3-carboxylation and C4-decarboxylation around the phosphoenolpyruvate–pyruvate–oxaloacetate node, which interconnect the TCA cycle with glycolysis. These reactions result in direct oxaloacetate formation or depletion. Carboxylation of phosphoenolpyruvate catalyzed by phosphoenolpyruvate carboxylase and that of pyruvate by pyruvate carboxylase contribute to its formation. Accordingly, decarboxylation of oxaloacetate catalyzed by phosphoenolpyruvate carboxykinase and oxaloacetate decarboxylase form phosphoenolpyruvate and

pyruvate, respectively. The carbon interconversion between the TCA cycle and glycolysis can also be achieved by malic enzyme which decarboxylates malate to form pyruvate (Sauer and Eikmanns 2005). As malic enzyme depletes malate, the precursor of oxaloacetate in the TCA cycle, this reaction indirectly results in the depletion of oxaloacetate. *C. glutamicum* possesses all these enzymes, while *E. coli* has no oxaloacetate decarboxylase. In both organisms, phosphoenolpyruvate carboxylase is encoded by the *ppc* gene, phosphoenolpyruvate carboxykinase by *pck*, and pyruvate carboxylase by *pyc* (Sauer and Eikmanns 2005). Very recently, the oxaloacetate decarboxylase coding gene *odx* of *C. glutamicum* has been characterized (Klaffl and Eikmanns 2010). As for malic enzyme, unlike *C. glutamicum* which only has one encoded by *malE* (Gourdon et al. 2000), *E. coli* possesses two isoforms respectively encoded by *sfcA* and *maeB* (Bologna et al. 2007).

14.2.2 The Terminal Pathway of L-Threonine

Starting from the building block of L-aspartate, the biosynthesis of L-threonine comprises five successive reactions sequencially catalyzed by aspartate kinase, aspartyl semialdehyde dehydrogenase, homoserine dehydrogenase, homoserine kinase and threonine synthase.

Firstly, L-asparte is phosphorylated to form L-aspartyl-P by aspartate kinase, the first key enzyme in the L-threonine terminal pathway which serves to direct the carbon flux into the aspartate family of amino acids. *E. coli* possesses three aspartate kinase isoenzymes, aspartate kinase I, II and III (Chassagnole et al. 2001; Viola 2001). Aspartate kinase I and II, the most and the least abundant isoforms, respectively, exist as a catalytic domain in the bifunctional enzymes, aspartate kinase I-homoserine dehydrogenase I and aspartate kinase II-homoserine dehydrogenase II, correspondingly encoded by the *thrA* and *metL* genes (Katinka et al. 1980). Aspartate kinase III is a monofunctional enzyme encoded by the *lysC* gene (Shiio and Miyajima 1969). In *C. glutamicum*, the known aspartate kinase is encoded by the *lysC* gene. Although the enzymes involved in the terminal pathway of L-threonine are believed to have no isoenzyme components, deletion of *lysC* was detrimental but not lethal to a *C. glutamicum* strain grown on minimal medium (Jetten et al. 1995).

Secondly, L-aspartyl-P is deoxidized to form L-aspartyl semialdhyde by aspartyl semialdehyde dehydrogenase encoded by the *asd* gene in both *E. coli* and *C. glutamicum* (Boy and Patte 1972; Cremer et al. 1988).

Thirdly, L-homserine is synthesized from the deoxidization reaction of L-aspartyl semialdhyde by homoserine dehydrogenase, the second key enzyme of the pathway that controls carbon flux towards L-homoserine synthesis at the branchpoint of L-aspartyl semialdehyde. In *E. coli*, as mentioned above, two isoforms of homoserine dehydrogenase are known, both of which are present as the catalytic domain in the bifunctional aspartate kinaseI-homoserine dehydrogenase I and aspartate kinase II-homoserine dehydrogenase II. In *C. glutamicum*, homoserine dehydrogenase is encoded by *hom* (Follettie et al. 1988).

The conversion of L-homoserine to L-threonine is performed by homoserine kinase and threonine synthase. Homoserine kinase phosphorylates L-homoserine to form L-homoserine-P which is then dephosphorylated by threonine synthase to produce L-threonine. Homoserine kinase is the third key enzyme of the pathway; it controls carbon flux towards L-threonine synthesis at the branchpoint of L-homoserine. In both *E. coli* and *C. glutamicum*, homoserine kinase is encoded by the *thrB* gene and threonine synthase is by *thrC* (Follettie et al. 1988; Theze and Saint-Girons 1974).

14.2.3 The Efflux System of L-Threonine

After synthesis in the cell, L-threonrine could be excreted into the medium by both passive diffusion and carrier-mediated export, the latter accounting for over 90 % of the total efflux in *C. glutamicum* (Palmieri et al. 1996). Five L-threonine permeases have been characterized in *E. coli* (Eggeling and Sahm 2003). They confer tolerance to high concentration of L-threonine to the producing strains. However, only three of them, RhtA, RhtB and RhtC separately encoded by genes *rhtA*, *rhtB* and *rhtC*, show activity in exporting L-threonine out of cell (Kruse et al. 2002; Livshits et al. 2003). RhtA belonging to the drug/metabolite transporter super family can export both L-threonine and L-homoserine. RhtB and RhtC belonging to the RhtB translocator super family are specific exporters for L-threonine (Diesveld et al. 2009; Eggeling and Sahm 2001). The activity of RhtC is higher than that of RhtB. *C. glutamicum* is assumed to be deficient in the export system of L-threonine (Debabov 2003). The only identified transporter showing activity in exporting L-threonine in this organism is ThrE encoded by the *thrE* gene. Nevertheless, ThrE has a low affinity with L-threonine and exports both L-threonine and L-homoserine (Simic et al. 2001).

14.2.4 Carbon Flux Depleting Pathways

The loss of available carbon flux for L-threonine biosynthesis is mainly through the L-lysine and L-methionine competing branches existing in the terminal pathway and its intracellular depletion towards L-isoleucine and glycine. The L-lysine branch at the nexis of L-aspartyl semialdehyde is initiated by dihydrodipicolinate synthase encoded by the *dapA* gene in both organisms (Velasco et al. 2002). The L-methionine branch at L-homoserine is initiated by the *metA* gene encoding homoserine succinyltransferase in *E. coli*, while by *metX* encoding homoserine acetyltransferase in *C. glutamicum* (Rückert et al. 2003). It should be noted that *C. glutamicum* utilizes novel split pathways for both L-lysine and L-methionine biosynthesis (Lee and Hwang 2003; Schrumpf et al. 1991). The depletion of L-threonine towards L-isoleucine is initiated by threonine dehydratase. Both *E. coli* and *C. glutamicum* possess two isoforms of threonine dehydratase encoded by the *ilvA* and *tdcB* genes

respectively (Kalinowski et al. 2003; Mockel et al. 1992; Umbarger and Brown 1957). The expression of *tdcB* would take place only under anaerobic conditions in *E. coli* (Umbarger 1973). The depletion of L-threonine towards glycine in *E. coli* can be initiated by two enzymes, namely threonine dehydrogenase encoded by the *tdh* gene (Bell and Turner 1976) and threonine aldolase encoded by the *ltaE* gene (Liu et al. 1998). However, only when the intracellular glycine is lacking will the threonine aldolase function as a compensatory force. In *C. glutamicum*, a side activity of serine hydroxymethyl transferase encoded by the *glyA* gene fulfills the function of cleaving L-threonine directly into glycine and acetaldehyde (Simic et al. 2002). The main substrate of this enzyme is L-serine with which the cleavage activity is 24-fold higher than with L-threonine.

14.3 Regulation of L-Threonine Biosynthesis

The biosynthesis of L-threonine is subjected to strict regulation due to its physiological importance in the cellular metabolism. A computational analysis of the global metabolic network of *E. coli* by Almaas et al. (2004) indicated that the L-threonine biosynthesis along with its conversion into glycine was a component of the high-flux backbone of metabolism. Hartman (2007) reported that the biosynthesis and uptake of L-threonine was of great significance for *Saccharomyces cerevisiae* cell to maintain stability, as L-threonine could be converted into glycine and subsequently initiate *de-novo* purine synthesis. Curien et al. (2009) found that in *Arabidopsis* L-threonine played an integrative role in regulating the metabolic distribution of L-aspartate.

14.3.1 The Regulation of Repression

In *E. coli*, the regulation of repression occurs in the expression of all the genes in the L-threonine terminal pathway: *thrA*, *thrB* and *thrC* by L-threonine and L-isoleucine in a covalent manner (Theze and Saint-Girons 1974); *metL* by L-methionine; *lysC* by L-lysine; *asd* by L-lysine, L-threonine and L-methionine in a multivalent manner (Boy and Patte 1972).

The *E. coli thrA*, *thrB* and *thrC* are sequencially clustered in the *thr* operon along with a leader sequence *thrL* containing 178 base pairs preceding *thrA*. The simultaneous repression of the expression of *thrA*, *thrB* and *thrC* by L-threonine and L-isoleucine can be ascribed to the transcriptional attenuation of the operon through *thrL* (Theze and Saint-Girons 1974). An internal region of *thrL* potentially encodes a short peptide with 8 threonine codons and 4 isoleucine codons, 11 of which are tandemly arranged. The downstream region of the tandem codons in *thrL* tends to form a stem and loop structure, either a terminator or an antiterminator depending on the availability of L-threonine and L-isoleucine in the cell. When L-threonine and

L-isoleucine are both in excess, the translation of the short peptide proceeds, resulting in the formation of a terminator structure, so the transcription of the *thr* operon is pre-terminated. By contrast, when L-threonine and L-isoleucine are lacking, translation of the short peptide stalls, leading to the formation of an antiterminator structure, so the transcription of the *thr* operon can continue. Mutation of a G insertion at position -37 upstream of *thrA* could cause derepression, probably through destabilizing the terminator structure (Gardner 1979; Gardner and Reznikoff 1978).

The repression of *E. coli lysC* expression by L-lysine is exerted through translational attenuation. The adjoining upstream of *lysC* resided a leading sequence that can form a complex secondary structure of 6 helixes via internal base pairing during transcription. When L-lysine is in excess, one of the helixes formed covers the Shine-Dalgarno sequence (SD sequence), preventing the binding of the ribosome to mRNA, so the translation of *lysC* can not be initiated. By contrast, when L-lysine is deficient, a different secondary structure forms, releasing the SD sequence, thus the translation of *lysC* can proceed (Grundy et al. 2003).

In *C. glutamicum,* the expression of *hom* and *thrB* is subjected to the repression by L-methionine. The two genes are clustered in one operon in the direction of 5'-*hom-thrB*-3' with an internal 10 bp non-coding space. Also resided in this operon is a long reversibly repeated sequence upstream of *hom,* which is apt to form a stem structure with a Gibbs free energy of −16.2 kJ/mol (Mateos et al. 1994). The repression might be a transcriptional attenuation-like regulation exerted through the leading sequence. Such types of regulation may need the assistance of some regulatory proteins (Henkin and Yanofsky 2002; Mateos et al. 1994). However, the transcription mode of *hom-thrB* operon remains unclear (Diesveld et al. 2009; Mateos et al. 1994), and the evidence of transcriptional attenuation-like regulation needs further investigation.

14.3.2 The Regulation of Inhibition

E. coli aspartate kinase I is subjected to feed-back inhibition by L-threonine. The inhibition is partial, and the inhibitory mechanism is allosteric and competitive with L-aspartate. Homoserine dehydrogenase I is also partially inhibited by L-threonine, but the inhibitory mechanism is non-competitive (Chassagnole et al. 2001). The bifunctional aspartate kinase I-homoserine dehydrogenase I is a homotetramer and each peptide chain contains two catalytic domains, with the kinase site residing on the N-terminal region and the dehydrogenase site residing on the C-terminal region. Between the two catalytic domains located an interphase region responsible for the allosteric regulation by L-threonine (Fazel et al. 1983). Although it was reported previously that the inhibition of homoserine dehydrogenase I was mediated through the aspartate kinase I domain (Truffa-Bachi et al. 1974), the subsequent research by James and Viola (2002) also indicated that the inhibition might be exerted by the interphase region. The mechanism of inhibition remains ambiguous without a clear picture of the natural structure of aspartate kinase I-homoserine dehydrogenase I.

E. coli aspartate kinase III is subjected to feed-back inhibition by L-lysine. The inhibition is complete (Chassagnole et al. 2001), and the inhibitory mechanism is

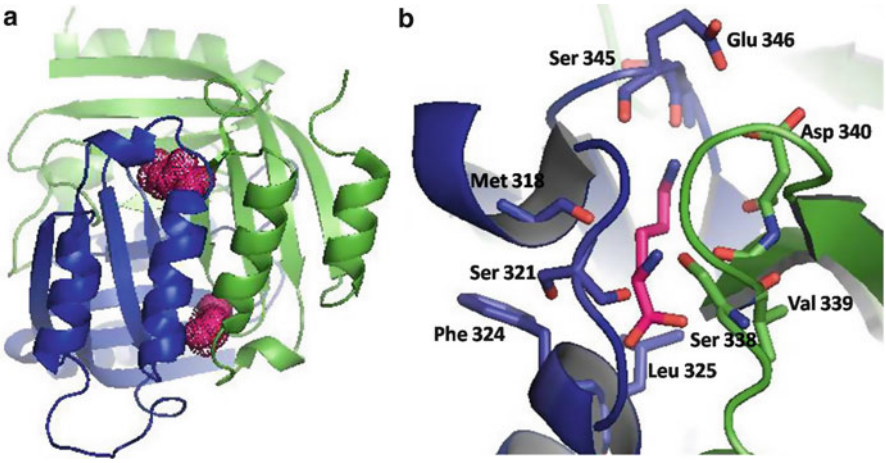

Fig. 14.2 L-lysine binding site in *E. coli* aspartate kinase III. (**a**) The dimer structure of the regulatory domains is shown. Regulatory domains from two different chains are shown in blue and green, respectively. The bound L-lysine molecules are shown in pink. (**b**) The bound L-lysine molecule and the amino acid residues involved in its binding are both shown in the manner of sticks. These models are built by using the PyMOL software, Protein Data Bank (accession number 2J0X) and the published information by Kotaka et al. (2006)

allosteric (Kotaka et al. 2006). The functional *E. coli* aspartate kinase III is a homodimer (Fig. 14.2a). The N-terminal region of each subunit functions as a catalytic domain, containing substrate binding sites for L-aspartate and ATP. The C-terminal region serves as a regulatory domain, containing two perpendicularly arranged ACT domains, ACT1 and ACT2. The ACT (Acronym for aspartate kinase, Chorismate mutase and TyrA) domain is a conserved structure responsible for the binding of small-molecule regulatory ligands found in functionally diverse proteins (Chipman and Shaanan 2001). The interface of the two ACT1 domains from different subunit shape binding sites for two L-lysine molecules (Fig. 14.2b). Mutations of T344M, S345L, T352I, all in the ACT1 domain, have been confirmed to be associated with partial L-lysine resistance of the enzyme. The ACT2 domains play the role of stabilizing the dimer structure and transmitting the L-lysine binding signal to the catalytic domain. The mechanism of inhibition is that the binding of L-lysine to the regulatory domains triggers tetramerization of two dimers and concomitant allosteric transition of the catalytic domains, resulting in blockage of the ATP binding site and eventually loss of activity. Furthermore, the allosteric transition disrupts one hydrogen bond at the L-aspartate binding site, which does not affect the substrate binding ability but may reduce the conversion rate (Kotaka et al. 2006).

The inhibition of *E. coli* homoserine kinase is complicated. It is inhibited by the substrates, L-homoserine at a concentration above 1 mM and ATP above 3 mM in a hypothetical "preferred order" manner, by L-threonine in a competitive manner, and by L-lysine in a non-competitive manner (Chassagnole et al. 2001).

In *C. glutamicum*, aspartate kinase is subjected to feed-back inhibition by L-lysine. The inhibitory mechanism is allosteric. *C. glutamicum* aspartate kinase is

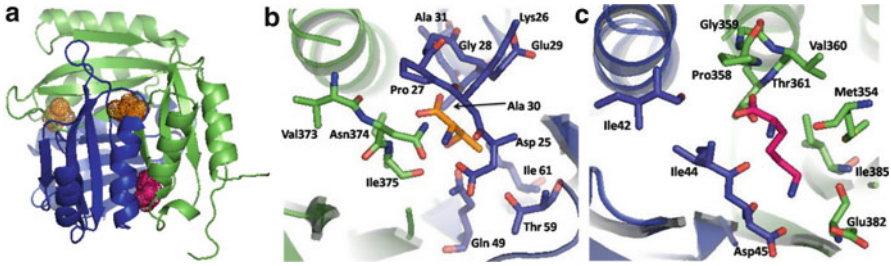

Fig. 14.3 L-threonine and L-lysine binding sites in *C. glutamicum* aspartate kinase. (**a**) The dimer structure of αβ-subunits is shown. The regulatory domain from α-subunit is shown in green and β-subunit is shown in blue. The bound L-threonine and L-lysine molecules are shown in orange and pink, respectively. (**b**) The bound L-threonine molecule and the amino acid residues directly involved in its binding are shown in the manner of sticks. (**c**) The bound L-lysine molecule and the amino acid residues directly involved in its binding are shown in the manner of sticks. These structural models are built by using the PyMOL software, Protein Data Bank (accession number 3AAW) and the published information by Yoshida et al. (2010)

a heterotetramer, comprised of two α subunits and two β subunits. As the two types of subunits are encoded by a single gene *lysC* with an in-frame sequence overlap (Kalinowski et al. 1990), the amino acid sequence of the β subunit is identical to 160 residues of the C-terminus of the α subunit. The N-terminal region of the α subunit functions as the catalytic domain, while its C-terminal region and the β subunit serve as the regulatory domain (Kato et al. 2004). Each regulatory domain contains two perpendicularly arranged ACT domains, ACT1 and ACT2 (Yoshida et al. 2007). Interaction of the β-subunit with the regulatory domain of the α-subunit causes the formation of four effector-binding sites, three of which are for the binding of two L-threonine and one L-lysine molecules (Fig. 14.3a). The L-threonine binding sites (Fig. 14.3b) are located in the interphase between ACT1 from the β-subunit and ACT2 from the α-subunit, and between ACT1 from the α-subunit and ACT2 from the β-subunit. The L-lysine binding site (Fig. 14.3c) is located in the interphase between ACT1 from the β-subunit and ACT2 from the α-subunit. The mechanism of inhibition may involve two steps. Firstly, the binding of L-threonine triggers dimerization of the β-subunit and the regulatory domain of the α-subunit. Then the binding of L-lysine triggers partial dissociation of the dimer and enables the interaction of the β-subunit with the catalytic domain of the α-subunit, resulting in an inactive conformation of the enzyme. Furthermore, L-lysine may also bind to the binding site for L-aspartate, which stabilizes the inactive conformation (Yoshida et al. 2010). Mutations in the regulatory domains of aspartate kinase were discovered in *C. glutamicum* strains with resistance to the L-lysine analog S-(2-aminoethyl)-l-cysteine (Yoshida et al. 2007).

C. glutamicum homoserine dehydrogenase is subjected to feed-back inhibition by L-threonine. The inhibition is almost complete when the concentration of L-threonine reaches 2 mM. The binding site for L-threonine is located in the C-terminus (Archer et al. 1991). Mutation of G378E conferred L-threonine resistance on homoserine

dehydrogenase (Reinscheid et al. 1991). The activity of the mutant was not affected by 25 mM L-threonine and was inhibited by 50 % when the concentration reached 100 mM. Furthermore, the residue 378 was confirmed to be essential for regulation as both the size and the charge of the amino acid at that position affected the enzyme's sensitivity to L-threonine. *C. glutamicum* homoserine kinase is inhibited by L-threonine with the concentration for half maximal inhibition of 25 mM (Colon et al. 1995). The inhibition is competitive and is relieved as the concentration of L-homoserine increases (Miyajima et al. 1968).

14.4 Metabolic Engineering for L-Threonine Production

The strategies recruited in the metabolic engineering for L-threonine production can be summarized as follows; (1) overexpressing the key enzymes coding genes of the biosynthesis pathway to condense carbon influx, (2) attenuating the competing branches to save more available precursors, (3) reducing intracellular depletion of L-threonine, (4) enhancing L-threonine secretion, (5) systems metabolic engineering. This section will focus on the effects of application of these strategies on L-threonine production in strains with almost defined genotypes.

14.4.1 Overexpressing the Genes of the Biosynthesis Pathway

Overexpressing the genes of the biosynthesis pathway, especially the deregulated ones encoding key enzymes, is usually the most productive strategy. Application of such strategy has achieved significant enhancements in L-threonine production. The priority of this strategy has been given to the genes of the L-threonine terminal pathway, and the outcome is better with mutated alleles coding deregulated key enzymes.

In *E. coli*, as *thrA*, *thrB*, and *thrC* are clustered in the *thr* operon, and aspartate kinase I encoded by *thrA* exists most abundantly among the three aspartate kinases, engineering of the L-threonine branch has been focused on this operon (Table 14.2). In most cases, the overexpressed *thr* operon contained a desensitized *thrA* gene. Introduction of a recombinant plasmid containing the *thr* operon from an L-threonine producer *E. coli* βIM4 into the very donor strain led to an increase in L-threonine production by threefold reaching 13.4 g/L (Miwa et al. 1983). Similarly, introduction of a recombinant plasmid containing the mutant $thrA_{442}BC$ operon into its donor strain *E. coli* MG442 also increased the L-threonine production from 8 to 18.4 g/L (Livshits et al. 2003). Recently, the wild-type *thr* operon and the mutant $thrA_{345}BC$ operon, both under their native promoters, were separately overexpressed on a high-copy number plasmid pMD19-T in the wild-type strain *E. coli* W3110. Overexpression of the *thr* operon and the $thrA_{345}BC$ operon both apparently increased the L-threonine production, from 0.036 to 2.590 and 9.223 g/L respectively (Zhang et al. 2009).

Table 14.2 Effects of metabolic engineering strategies on L-threonine production

Strategies	Strains	Increment[a] (fold)	Yield (g/L)	Reference
Overexpressing genes of L-threonine biosynthesis pathway	*E. coli*			
	βIM4(pBR322-*thrA*^{r})	3	13.4	Miwa et al. (1983)
	MG422(pAYC32-*thrA*^{r}*BC*)	1.3	18.4	Livshits et al. (2003)
	W3110(pMD19-*thrLABC*)	71	2.59	Zhang et al. (2009)
	W3110(pMD19-*thrLA*^{r}*BC*)	255	9.223	Zhang et al. (2009)
	C. glutamicum			
	DM368-3(pEK-*hom*^{r}-*thrB*)	1.13	1.7	Eikmanns et al. (1991)
	MH20-22B-(*hom*^{r}-*thrB*)^3	>77	7.7	Reinscheid et al. (1994)
	ATCC21799(pGC42)	>118	11.8	Colon et al. (1995)
Reducing the intracellular depletion of L-threonine	*C. glutamicum*			
	DM368-2*glyA'*	0.5	1.3	Simic et al. (2002)
	DM1800*ilvA*^{M} (pET-T18*hom*^{r}-*thrB-thrE*)	0.6	4	Diesveld et al. (2009)
Enhancing L-threonine efflux	*E. coli*			
	MG422(pTrc99A-*rhtB*)	1.4	–	Kruse et al. (2002)
	MG422(pTrc99A-*rhtC*)	2	–	Kruse et al. (2002)
	MG422(pTrc99A-$thrE_{Cg}$)	2.9	–	Kruse et al. (2002)
	MG422 *rhtA23*(pAYC32-*thrA*^{r}*BC*)	1	36.3	Livshits et al. (2003)
	C. glutamicum			
	MH20-22B-(*hom*^{r}-*thrB*)^3 (pEC-T18mob2-*thrE*)	0.05	8.1	Simic et al. (2002)
	DM368-2*glyA'*(pEC-T18mob2-*thrE*)	0.15	1.5	Simic et al. (2002)
	DM368-3(pEKEx2-$rhtC_{Ec}$)	3.11	3.7	Diesveld et al. (2009)
	DM1800(pET-T18*hom*^{r}-*thrB-thrE*) (pEKEx2-$rhtC_{Ec}$)	1.56	6.4	Diesveld et al. (2009)
Systems metabolic engineering	*E. coli*			
	TH28C (pBRThrABCR3)	–	82.4	Lee et al. (2007)
	MDS-205	–	40.1	Lee et al. (2009)

[a]Approximate value

In *C. glutamicum*, the strategy of co-expressing the three key enzymes coding genes, *lysC*, *hom* and *thrB*, has been confirmed feasible. Overexpression of *lysC* is the prerequisite for L-threonine production as aspartate kinase controls the total carbon-influx into the biosynthesis pathways of aspartic family of amino acids. Therefore *C. glutamicum* strains carrying a $lysC^r$ on the chromosome were mostly used as base strains for metabolic engineering (Colon et al. 1995; Eikmanns et al. 1991; Reinscheid et al. 1994). The mutation of S301Y in chromosomal *lysC* which conferred S-(2-aminoethyl)-l-cysteine-resistance on aspartate kinase additionally enhances the downstream *asd* expression, probably due to the accidental formation of a stronger internal promoter (Kalinowski et al. 1991). Although *C. glutamicum* ($lysC^r$) strains are L-lysine producers due to the absence of regulation for the L-lysine branch in this organism (Wittmann 2010), overexpression of the hom^r-*thrB* operon redirects the carbon flux from L-lysine branch into L-threonine branch, leading to the accumulation of both L-threonine and L-homoserine (Colon et al. 1995; Reinscheid et al. 1994). Introduction of a recombinant plasmid containing the hom^r-*thrB* operon into *C. glutamicum* DM368-3 (AEC^r, AHV^r) doubled L-threonine production from 0.8 to 1.7 g, meanwhile reduced L-lysine production from 1.3 to 0.018 g/L (Eikmanns et al. 1991). Integration of three additional copies of the hom^r-*thrB* operon into the chromosome in the L-lysine producing strain *C. glutamicum* MH20-22B resulted in the production of 7.7 g/L L-threonine and 2.5 g/L L-homoserine and a significant decrease in L-lysine production from 30.4 to 8.5 g/L (Reinscheid et al. 1994). Further increasing the expression of *thrB* to a higher level than that of *hom* efficiently circumvents L-homoserine accumulation (Colon et al. 1995). Introduction of a recombinant plasmid, on which hom^r was expressed constitutively under its native promoter and *thrB* was expressed inductively under the P_{tac} promoter, into *C. lactofermentum* ATCC21799 (AEC^r) led to an L-threonine production of 11.8 g/L with no L-homoserine accumulation, and a dramatic reduction in L-lysine production from 22.0 to 0.8 g/L (Colon et al. 1995).

14.4.2 Enhancing L-Threonine Efflux

When L-threonine accumulates to a certain level in the cell, the prompt secretion becomes limiting for production. Even for Gram-negative *E. coli*, whose cell envelope is not considered as a permeation barrier for amino acids, the intracellular L-threonine concentration exceeded the one detected in medium throughout the fermentation course, with a tenfold excess observed in early stages (Kruse et al. 2002). Gram-positive *C. glutamicum* possesses a characteristic cell wall structure, containing an outer layer of mycolic acids (Eggeling and Sahm 2001), the amino acid efflux in *C. glutamicum* is therefore significantly impaired. High intracellular concentrations of L-threonine down-regulate the biosynthesis enzymes in a feed-back manner, increase the precursor availability for the depletion pathway, and even inhibit cell growth. The strategy of overexpressing the specific permease-coding genes, $rhtA_{Ec}$, $rhtB_{Ec}$, $rhtC_{Ec}$ *and* $thrE_{Cg}$, has been confirmed effective in accelerating L-threonine secretion and thus contributing to an increased production.

In *E. coli*, individual overexpression of $rhtB_{Ec}$, $rhtC_{Ec}$ and $thrE_{Cg}$ on an episomal plasmid pTrc99A in strain MG422 increased the L-threonine production by 140, 200 and 290 %, respectively, as compared with the control strain MG422 (pTrc99A) (Kruse et al. 2002). In another case, strengthening the transcription of *rhtA* in strain MG422 (pAYC32-*thrA*^{r}*BC*), by introducing a point mutation (G→A) one base upstream the start codon of *rhtA* on the chromosome, increased L-threonine production from 18.4 to 36.3 g/L (Livshits et al. 2003).

As in the case of *C. glutamicum*, introduction of a recombinant plasmid containing $thrE_{Cg}$ into strain DM368-2*glyA'* led to an increase in L-threonine production from 1.3 to 1.5 g/L and a reduction in glycine accumulation. When the same recombinant plasmid was introduced into strain MH20-22B-(*hom*^{r}*-thrB*)[3], L-threonine production increased from 5.8 to 8.1 g/L with reduced accumulation of L-lysine, glycine and L-isoleucine (Simic et al. 2002). Overexpression of $rhtA_{Ec}$, $rhtC_{Ec}$ and $yeaS_{Ec}$ in different *C. glutamicum* strains also has a positive effect on L-threonine production. The best result was obtained when $rhtC_{Ec}$ gene was expressed on an episomal plasmid pEKEx2, which increased L-threonine production in strain DM368-3 (AEC^r, AHV^r) from 0.9 to 3.7 g/L and in DM1800 (pET-T18*hom*^{r}*-thrB-thrE*) from 4 to 6.4 g/L without L-homoserine accumulation (Diesveld et al. 2009).

14.4.3 Reducing the Intracellular Depletion of L-Threonine

The strategy of reducing L-threonine depletion towards L-isoleucine and glycine in the cell has been applied in both *E. coli* and *C. glutamicum*, and confirmed effective in increasing L-threonine production and reducing by-products formation. Reducing intracellular L-threonine conversion towards L-isoleucine in *E. coli* with genotype of *relA*$^+$ also results in activation of the *thr* operon via a "stringent response" mechanism in addition to a derepression effect (Debabov 2003). Lee et al. (2007) attenuated L-threonine depletion in *E. coli* by introducing a mutation of C290T into the chromosomal *ilvA* gene and deleting the chromosomal *tdh* gene. Simic et al. (2002) weakened the conversion of L-threonine to glycine in *C.glutamicum* DM368-2 (*lysC*^{r}, *hom*^{r}) by down-mutating the promoter of *glyA*. As a result, L-threonine production was increased to 1.3 g/L, and glycine accumulation was reduced from 0.5 to 0.3 g/L in minimal media. Similarly, Diesveld et al. (2009) cut down the conversion of L-threonine to L-isoleucine in *C.glutamicum* DM1800-T by down-mutating the promoter of *ilvA*, which increased L-threonine production from 2.5 to 4 g/L.

14.5 Systems Metabolic Engineering

Application of systems metabolic engineering gives rise to breakthroughs in strain construction. So far, a few *E. coli* L-threonine hyper-producers have been constructed by this means, and the total conversion rate of glucose to L-threonine and biomass approximates to the predicted theoretical values.

The most typical case of this strategy is the construction of *E. coli* TH28C (pBRThrABCR3) by Lee et al. (2007). A *lacI*$^-$ mutant strain of *E. coli* W3110 was used as the base strain so that promoters such as P_{tac} and P_{trc} could initiate transcription constitutively. First, the carbon influx for L-threonine biosynthesis was condensed by three steps. The feed-back inhibitions of aspartate kinase I and III were released through site-directed mutagenesis of C1034T in *thrA* and C1055T in *lysC* in chromosome; the feed-back repression to the chromosomal *thr* operon was released by substituting its native promoter with P_{tac}; and the deregulated *thr* operon was overexpressed on an episomal vector. Secondly, the carbon depleting pathways were attenuated. The production of L-lysine was blocked by deleting *lysA* encoding the last enzyme involved in L-lysine branch; the L-methionine branch was shut down by deleting *metX*; the depletion of L-threonine towards glycine was reduced by deleting *tdh*; and the depletion of L-threonine towards L-isoleucine was ablated by down-regulating threonine dehydratase activity through mutagenesis of C290T in *ilvA*. Thirdly, the availability of oxaloacetate was increased by enhancing the transcription of the *ppc* gene through substituting its native promoter with a stronger promoter P_{trc}, and enhancing the glyoxylate shunt through deleting the *iclR* gene encoding the repressor of isocitrate lyase and malate synthase. Fourthly, the efflux of L-threonine was enhanced by deleting the *tdcC* gene encoding an uptake-carrier and overexpressing *rhtA*, *rhtB* and *rhtC* on the same episomal vector as for the *thr* operon. Both the third and fourth steps were performed according to the transcriptome and *in silico* flux analysis. Finally, the production of acetate was attenuated by strengthening the transcription of the *acs* gene encoding acetyl-CoA synthetase through substituting its native promoter with the stronger promoter P_{trc}. The constructed strain TH28C (pBRThrABCR3) could produce 82.4 g/L L-threonine in 50 h' fed-batch fermentation. The L-threonine/glucose conversion rate was 39.3 %. No lactate was formed and the accumulation of acetate was 2.35 g/L.

In another successful case, Lee et al. (2009) constructed a plasmid-free L-threonine hyper-producer of *E. coli*, MDS-205, from a reduced-genome strain *E. coli* MDS42. In MDS-205, the *thr* operon together with its native promoter was substituted with the L-threonine resistant *thrA*^{r}*BC* operon under the stronger promoter P_{tac} on the chromosome, and the *lacI* gene encoding the P_{tac} repressor was deleted. The *tdh* gene was deleted to reduce the L-threonine depletion. The L-threonine uptake facilitator-coding genes, *tdcC* and *sstT*, were substituted with a mutant exporter gene *rhtA23*, which not only blocked the L-threonine uptake but also enhanced its export. The constructed strain showed robust growth and better performance in high cell-density fermentations. It produced 40.1 g/L L-threonine in 30 h' batch-fermentation with an L-threonine/glucose conversion rate of 39.3 % (Lee et al. 2009).

14.6 Conclusion

With the identification of more genes encoding the enzymes involved in L-threonine biosynthesis and the unveiling of the molecular mechanisms of regulation, metabolic engineering for L-threonine production has achieved preliminary success.

Systems metabolic engineering that globally modifies the metabolic network has shown its superiority in effectiveness over simply engineering one or two properties of the strain. Information collected from "Post genome study" on traditionally-bred L-threonine producers contributes to completing our knowledge of cellular metabolism and L-threonine biosynthesis (Kim et al. 2004; Lee et al. 2003, 2007). Knowledge gained from kinetic studies on biosynthesis enzymes reveals the essential biochemical characteristics of L-threonine biosynthesis (Chassagnole et al. 2001; Curien et al. 2009; Rodríguez-Prados et al. 2009). Evolution of methods and tools (Pátek and Nešvera 2011; Tan et al. 2012; Tyo et al. 2009; Xu et al. 2010) accelerates the engineering process. Fast development of systems biology and synthetic biotechnology enables genome-wide reprogramming of cells (Feist et al. 2007; Feist and Palsson 2008; Oberhardt et al. 2009; Shinfuku et al. 2009; Tyo et al. 2010). Thus it is promising to engineer microbes into more productive platforms for L-threonine production.

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