Metabolic Control of Aspartate-Dervied Amino Acid Production in *Corynebacterium glutamicum*

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Introduction

Corynebacterium has played a major role in the industrial production of primary metabolites, such as amino acids and nucleotides (Kinoshita, 1985). Increasing and optimizing the final yield of metabolites through strain development and improvement has long been a major interest in food and feed industry. Traditionally, strain improvement was achieved by classical mutagenesis using chemical mutagens and selecting for the strain which produces increased and optimized amount of metabolites (Liebl, 1991). Even though this approach has been used successfully to increase the final yield of certain amino acids, random mutagenesis typically leads to the accumulation of undesirable secondary mutations which limit further improvement of the strain.

The advent of recombinant DNA technology opened a new possibility in strain development and manipulation (Martin, 1989; Schäfet al., 1990; Wohlleben et al., 1992; Yoshihama et al., 1985). The recent development and use of genetic and molecular biological tools for the industrially important *Corynebacterium* have made significant progress in our understanding of biosynthetic pathways of several amino acids. This new information has been applied successfully to the development and improvement of *Corynebacterial* strains (Jetten et al., 1993).

In this report, I will briefly explore the recent progresses in the metabolic engineering of *Corynebacterium* and related species, especially *Corynebacterium glutamicum*. I will focus mostly on the biosynthesis of aspartate family of amino acids, such as lysine and threonine. The information on the biosynthesis of other members of aspartate family of amino acids, such as methionine and isoleucine, is still very limited. Therefore, they will not be discussed here.

Corynebacterium

Corynebacterium belongs to the *Actinomycetes* subdivision of Gram-positive eubacteria which are represented by high GC content (Liebl, 1991). They are widely distributed in nature and include very diverse group of bacteria. These microorganisms are typically identified by their "club" (or "V") shape even though it is not unique only to *Corynebacteria*. The closely related organisms include *Mycobacterium, Nocardia*, and *Rhodococcus* (called CMN group) (Barksdale, 1970).

Modern taxonomic schemes based on ribosomal RNA sequence, biochemical properties, cell wall structure and component, and DNA base ratios have identified some species previously classified in the genera of *Arthrobacter, Brevibacterium,* and *Microbacterium* as the true member of *Corynebacterium*. Based on these criteria it has become evident that glutamic acid-producing *B. ammoniagenes, B. divaricatum, B. flavum,* and *B. lactofermentum* are almost synonymous to *C. glutamicum,* if not identical. In this article the strains

originally referred to as *B. flavum* and *B. lactofermentum* will be used synonymous to *C. glutamicum.*

Genetic tools for *Corynebacterium*

A series of Corynebacterial plasmids which can be used for the construction of cloning vectors have been identified in many *Corynebacterial* species. The plasmids ranged from approximately 3 kb to more than 100 kb in size and were mostly cryptic in function. Some of the naturally occurring cryptic plasmids, such as the 4.4 kb plasmid pBL1 from *B. lactofermentum* (Santamaria et al., 1984), 3.0 kb plasmid pSR1 from *C. glutamicum* (Yoshihama et al., 1985), pCC1 from *C. callunae* (Sandoval et al., 1984), and 4.9 kb plasmid pGA1 from *C. glutamicum* (Sonnen et al., 1991) have been used successfully to construct cloning vectors, of which some of them were *E. coli-Corynebacterium* shuttle vectors. The plasmids were chosen primarily due to their small size. The shuttle vectors typically contain two different origins of replication: one functions in *E. coli* and the other functions in *Corynebacterium.*

Some of the genes which confer resistance to antibiotics and were originated from *E. coli* and *Bacillus* and related organisms turned out to be nonideal for use as selectable markers in *Corynebacterium* due to the intrinsic resistance of the bacterium to some of the antibiotics and/or low level expression of some of the markers in that organism. Among the widely used markers, kanamycine resistance which was originated from transposon Tn5 appeared to be the most suitable as a selectable marker and has been widely used in the construction of *E. coli-Corynebacterium* shuttle vectors (Martin et al., 1987). Bleomycin has been described recently as an effective marker for Corynebacteria (Guerrero et al., 1992).

Corynebacterium shows wide acceptance of foreign DNA. Genes from *E. coli, Bacillus subtilis,* and *Streptomyces* are expressed in *Corynebacteria* to certain extent. Three major techniques have been used for the introduction of foreign DNA into *Corynebacterium;* protoplast transformation, electroporation, and transconjugation (Liebl, 1991). Electroporation (Bonamy et al., 1990) has been shown to be more efficient and convenient than protoplast transformation in *Corynebacterium.* A conjugal transfer of mobilizable plasmid has been developed more recently and used successfully for gene disruption and replacement (Gubler et al., 1993; Peters-Wendisch et al., 1993; Schäfer et al., 1990).

Central carbon metabolism

The aspartate family of amino acids derive their carbon from oxaloacetate (OAA), which, as an intermediate of the Kreb's cycle, is constantly replenished by anaplerotic enzyme reactions (Fig. 1). In prokaryotes, several enzymes are involved in the biosynthesis of OAA. These include phosphoenolpyruvate (PEP) carboxylase (Ozaki and Shiio, 1969), PEP carboxykinase (Jetten and Sinskey, 1993), pyruvate carboxylase (Tosaka et al., 1979), and the enzymes of the glyoxylate cycle (Ozaki and Shiio, 1968). Recent studies involving the expression of amino acid biosynthetic genes and metabolic flux analysis based on the concentration of metabolites during batch fermentation indicated that the regulation of the PEP branchpoint, where PEP is either converted to pyruvate by pyruvate kinase or carboxylated to OAA by PEP carboxylase, is the limiting factor in lysine production (Shiio et al., 1990; Vallino and Stephanopolous, 1993). In addition, PEP caboxylase activities from *C. glutamicum* and *C. flavum* were strongly inhibited by aspartate suggesting a role in carbon flow to aspartate (Mori and Shiio, 1985). Since the disruption of *ppc* gene encoding PEP carboxylase did not affect lysine yield or growth, it has been suggested that other PEP or OAA converting enzymes may play an important role in the regulation of carbon flux in *C. glutamicum*

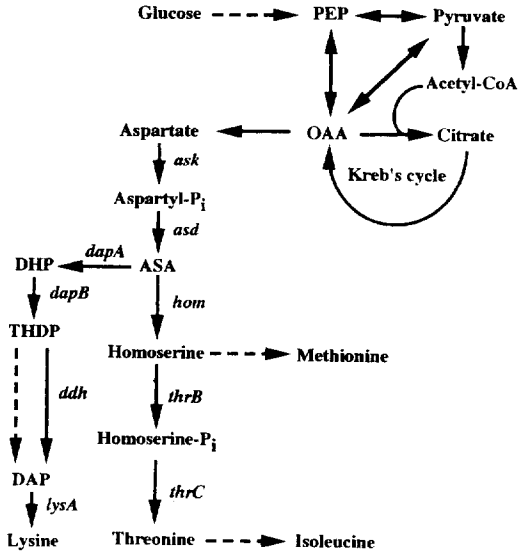

Fig. 1. Biosynthetic pathway of aspartate family of amino acids in *C. glutamicum*. Multiple steps are indicated by dashed lines. Abbreviations: ASA, aspartate; DHP, dihydrodipicolinate; OAA, oxaloacetate; PEP, phosphoenolpyruvate; THDP, tetrahydrodipicolinate.

(Gubler et al., 1993; Peters-Wendisch et al., 1993). PEP carboxykinase which catalyzes the interconversion of PEP and OAA is not inhibited by aspartate in the OAA forming reaction, indicating that the enzyme may play more important role in gluconeogenesis (PEP-forming reaction) rather than in amino acid biosynthesis (Jetten and Sinskey, 1993).

Common pathway

The aspartate family of amino acids derive most or all of the carbon atoms from aspartate (Fig. 1). The biosynthetic pathways of lysine and threonine from aspartate have been well characterized. The first two steps, the synthesis of aspartyl-phosphate from aspartate by aspartokinase and subsequent conversion to aspartate semialdehyde by aspartate-semialdehyde dehydrogenase, are common for the aspartate family of amino acids. Unlike *E. coli* and *Bacillus* which contain multiple isoenzymes, *C. glutamicum* appears to contain only one aspartokinase which is almost completely feedback inhibited by excess amount of lysine and threonine (Kase and Nakayama, 1974; Tosaka and Takinami, 1978): threonine or lysine alone inhibits only 10% of the activity. Thus, deregulation of aspartokinase (encoded by *ask* or *lysC*) typically results in increase in lysine or threonine production (see below). The mutation responsible for the deregulation was determined in *C. flavum* and *C. glutamicum*. The mutation caused single amino acid change at its C-terminal region of the protein (Follettie et al., 1993; Jetten et al., 1993).

Threonine

The biochemical reactions leading to the biosynthesis of threonine in *Corynebacterium* are identical to that of *E. coli* and *Bacillus*, even though the genetic organization of the genes and biochemical activities of the enzymes involved show significant differences.

The threonine-specific biosynthetic pathway consists of three steps. The first step, the conversion of aspartate semialdehyde to homoserine, is catalyzed by homoserine dehydrogenase (encoded by *hom*). The carbon preferentially flows to the direction of homoserine (as compared to dihydrodipicolinate of lysine biosynthetic pathway) due to high substrate affinity and substrate conversion rate of the homoserine dehydrogenase relative to the dihydrodipicolinate synthase for the common substrate aspartate semialdehyde: the specific activity of homoserine dehydrogenase is 15-fold greater than that of dihydrodipicolinate synthase (Miyajima et al., 1968). The activity of homoserine dehydrogenase is highly sensitive to the allosteric inhibition by threonine (Miyajima and Shiio., 1970). Unlike homoserine dehydrogenase, the activity of homoserine kinase (encoded by *thrB*) which catalyzes the next step, the conversion of homoserine to

homoserine-phosphate, is not affected by threonine. The final step, the formation of threonine from homoserine-phosphate, is catalyzed by threonine synthase (encoded by *thrC*). Unlike *E. coli, thrC* is expressed independently of the other threonine-specific genes (Han et al., 1990).

Typically, threonine-overproducing strains are made by deregulating homoserine dehydrogenase making it insensitive to the feedback inhibition by threonine. Studies on the deregulated homoserine dehydrogenases with altered C-terminus indicated that the C-terminus of the enzyme might be involved in the allosteric response by threonine (Archer et al., 1991; Reinscheid et al., 1991). Introduction of multiple copies of *hom* (or deregulated) and *thrB* genes preferentially shifted carbon flow toward threonine biosynthetic pathway resulting in decrease in lysine production (Eikmanns et al., 1991, Jetten et al., 1993). As expected, amplification of deregulated homoserine dehydrogenase almost completely shut down lysine production. However, the sum of threonine and lysine produced by these recombinant strains was almost unaffected as compared to the parental strain indicating that carbon flow into the common aspartate pathway is the real limiting factor. The amplification of *thrC* did not appear to affect neither threonine nor lysine production even though the activity of threonine synthase was increased by several fold. Based on these observations it became clear that the limiting factor in the overproduction of threonine is the carbon flux into the aspartate pathway.

Lysine

The biosynthesis of lysine in *C. glutamicum* is controlled at the level of aspartokinase. There is no apparent regulation of the enzymes in synthesis and activity specific to lysine-branch of the pathway. The flow of carbon at aspartate semialdehyde branch point from which lysine specific pathway is separated depends on the activity of homoserine dehydrogenase (Miyajima and Shiio, 1970). In the presence of excess amount of th reonine, the activity of homoserine dehydrogenase is feedback inhibited directing the flow of carbon to dihydrodipicolinate, the first intermediate in lysine specific pathway. This results in the increased synthesis of lysine which in turn feedback inhibits the activity of aspartokinase. This effectively regulates overall synthesis of aspartate family of amino acids.

The classical approach of selecting a lysine overproducing strain has relied upon the use of lysine analogs, such as S-(2-aminoethyl)-L-cysteine (Schrumpf et al., 1992; Tosaka and Takinami, 1978). A class of mutant strains selected for the resistance to the toxic analog in the growth medium apparently overproduced lysine typically due to the presence of the aspartokinase which is no longer feedback regulated by lysine and its analog. Consistent with this notion, expression of cloned deregulated aspartokinase in wild type background alone was sufficient to produce high level of lysine (Cremer et al., 1991). Overproduction of aspartate alone by the introduction of *aspA* (encodes aspartase) gene from *E. coli* only marginally increased lysine production indicating that the size of aspartate pool is not the limiting factor (Menkel et al., 1989). C. *glutamicum* does not contain *aspA* gene which converts fumarate into aspartate and allows gro wth on fumarate. Overexpression of dihydrodipicolinate synthase alone also achieved lysine overproduction but to a less extent (Cremer et al., 1991). Overexpression of other enzymes in lysine biosynthetic pathway did not affect lysine production indicating that of the six enzymes of diaminopimelate dehydrogenase pathway that convert aspartate to lysine aspartokinase and dihydrodipicolinate synthase are responsible for the overall flow control.

Typically, effective excretion of lysine is achieved by deregulating the aspartokinase. This results in increased intracellular concentration of

lysine which appears to be responsible for lysine excretion. The mechanism by which this is achieved is unknown. Recently significant progress has been made toward understanding the mechanism of lysine export across the membrane (Bröer and Krämer, 1991). The export process was modulated by the membrane potential, lysine gradient, and pH gradient. The increased lysine secretion by some production strains appears to be caused by additional mutation in one or more of membrane transport components in addition to the deregulated aspartokinase (Schrump et al., 1992).

Overall control

The overall regulation of aspartate family of amino acids synthesis in *C. glutamicum* is modulated by the relative specific activities of the major branch point enzymes and feedback inhibition of the key enzymes by the end product. Lack of multiple isoenzymes in *C. glutamicum* shows differences in control mechanism relative to *E. coli* and *Bacillus*. Increased synthesis of threonine by the preferential flow of carbon through the threonine and methionine branch of the pathway due to higher specific activity of homoserine dehydrogenase over dihydrodipicolinate synthase for the common substrate aspartate semialdehyde results in the feedback inhibition of homoserine dehydrogenase by threonine. Increased synthesis of methionine represses expression of homoserine dehydrogenase at the level of transcription presumably by attenuation mechanism (Follettie et al., 1988). Decreased synthesis and activity of homoserine dehydrogenase due to excess amount of threonine and methionine channels carbon through the lysine biosynthetic pathway resulting in the increased synthesis of lysine which in turn inhibits the activity of aspartokinase in conjunction with threonine controlling the overall flow of carbon. The concerted action of these regulatory mechanisms controls the flow of carbon into the pathway and ensures the adequate supply of the amino acids.

Conclusion

Recent advances in molecular biology allowed development of new genetic tools for the industrially important *Corynebacterium*. The use of these new genetic tools greatly increased our understanding on biochemistry and genetics of the genes and proteins involved in the amino acid biosynthetic pathways. Unlike classical approach of strain improvement and development which makes both quantitative and qualitative analysis of the effect of mutational change difficult, recombinant DNA technique allows more defined control of the target genes and enzymes. This not only significantly improved our understanding on the nature of the mutations obtained by classical approach but also opened a new chapter in the way new strains are constructed.

The general goal of this new approach is to design a novel pathway which allows efficient flow of precursors maximizing the final yield of the metabolite. This has been employed in the construction of amino acid overproducing strains especially for lysine and threonine. Amplification of enzymes by the introduction of multiple copies of the gene readily identified rate limiting steps and facilitated construction of improved strain. Based on several studies, the importance of central carbon metabolism has been revealed in the biosynthesis of aspartate family of amino acids. Isolation of the genes and identification of the enzymatic activities of the central carbon metabolism will be necessary to measure the contribution of this pathway to amino acid biosynthesis and to construct metabolically improved strain.

Even though significant progress has been made in understanding the organization of genes and involvement of enzymes, still little is known about the regulatory mechanisms of Corynebacterial gene expression. The availability of this

information will greatly facilitate the fine control of gene expression. The recent surge of information on the genetics and biochemistry of amino acid synthesis in Corynebacterium will eventually lead to the construction of industrially important organisms not only for amino acids but also for other compounds.

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