Amplification of three threonine biosynthesis genes in *Corynebacterium glutamicum* and its influence on carbon flux in different strains

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Summary. The *hom-thrB* operon (homoserine dehydrogenase/homoserine kinase) and the *thrC* gene (threonine synthase) of *Corynebacterium glutamicum* ATCC 13032 and the *homFBR* (homoserine dehydrogenase resistant to feedback inhibition by threonine) alone as well as *homFBR-thrB* operon of *C. glutamicum* DM 368-3 were cloned separately and in combination in the *Escherichia coli/C. glutamicum* shuttle vector pEK0 and introduced into different corynebacterial strains. All recombinant strains showed 8- to 20-fold higher specific activities of homoserine dehydrogenase, homoserine kinase, and/or threonine synthase compared to the respective host. In wild-type *C. glutamicum*, amplification of the threonine genes did not result in secretion of threonine. In the lysine producer *C. glutamicum* DG 52-5 and in the lysine-plus-threonine producer *C. glutamicum* DM 368-3 overexpression of *hom-thrB* resulted in a notable shift of carbon flux from lysine to threonine whereas cloning of *homFBR-thrB* as well as of *homFBR* in *C. glutamicum* DM 368-3 led to a complete shift towards threonine or towards threonine and its precursor homoserine, respectively. Overexpression of *thrC* alone or in combination with that of *homFBR* and *thrB* had no effect on threonine or lysine formation in all recombinant strains tested.

Introduction

L-Threonine is one of the essential amino acids and, after lysine and methionine, in many cases the next limiting amino acid in animal and human foodstuffs (Kleemann et al. 1985). Therefore, there is an interest in microbial L-threonine production for food supplementation. Until a few years ago microbial threonine production was primarily dependent on mutant strains of *Serratia marcescens*, or of the coryneform species *Corynebacterium glutamicum*, *Brevibacterium flavum*, or *B. lactofermentum*, which were all obtained by classical mutagenesis (Kleemann et al. 1985; Kinoshita 1985). Only recently have powerful cloning systems been developed for coryneform bacteria (for a review see Martin et al. 1987) thus opening the possibility of studying the molecular biology of these organisms and of applying recombinant DNA techniques for strain development and for overcoming possible limiting steps in amino acid production.

L-Threonine biosynthesis from aspartate in coryneform bacteria has been well characterized. It consists of five enzymatic steps (Fig. 1), the initial two being common in threonine and lysine biosynthesis. In contrast to *E. coli* only one aspartate kinase is present (Shiio et al. 1970). This enzyme is feedback inhibited when both lysine and threonine are present in excess (Shiio and Miyajima 1969). Homoserine dehydrogenase (HDH) is feedback inhibited by threonine and both HDH and homoserine kinase (HK) are weakly repressed by methionine (Miyajima et al. 1968; Miyajima and Shio...
1970, 1971). The other two enzymes involved in L-threonine biosynthesis of coryneform bacteria seem not to be subjected to inhibition or repression (Miyajima et al. 1968; Miyajima and Shioj 1971).

Recently, the organization of the hom, thrB, and thrC genes coding for HDH, HK and threonine synthase (TS), respectively, from C. glutamicum (Katsumata et al. 1986; Follettie et al. 1988; Peoples et al. 1988; Han et al. 1990) and from B. lactofermentum (Mateos et al. 1987a, b) have been described. Also, attempts have been made to improve L-threonine production by amplification of homFBR-thrB coding for HDH released from feedback inhibition and HK in mutant strains of C. glutamicum or B. lactofermentum (Katsumata et al. 1986; Morinaga et al. 1987) or by amplification of the E. coli threonine operon in a B. flavum mutant strain (Ishida et al. 1989).

In order to obtain information on the limiting steps in carbon flux to threonine and in order to construct a well-defined recombinant threonine-producing strain we studied the separate and/or combined amplification of the threonine biosynthesis genes hom, homFBR, thrB, and thrC and its influence on amino acid formation in wild-type, in a lysine-producing strain, and in a lysine- and threonine-producing strain of C. glutamicum.

Materials and methods

Strains, plasmids, and media.

All bacterial strains and plasmids used in this study are listed in Table 1. The E. coli CGSC strains were obtained from Bachmann (New Haven, Conn., USA). The minimal medium used for C. glutamicum contained per litre: 5 g (NH₄)₂SO₄, 5 g urea, 0.5 g KH₂PO₄, 0.5 g K₂HPO₄, 20.9 g 3-(N-morpholino)-propane-sulfonic acid (MOPS), 0.25 g MgSO₄·7H₂O, 10 mg CaCl₂·2H₂O, 10 mg MnSO₄·H₂O, 10 mg FeSO₄·7H₂O, 1 mg ZnSO₄·7H₂O, 0.2 mg CuSO₄, 0.2 mg biotin. The pH was adjusted to 6.5 and 4% (w/v) glucose was added after sterilization. For determination of amino acid production the (NH₄)₂SO₄ concentration was increased to 20 g/l, urea and MOPS were omitted, and 20 g/1 CaCO₃ was added after sterilization. The pH was adjusted to 7.0, 0.25 mM NADPH, and 2.5 mM aspartate semialdehyde as substrate. The activity was determined by following the decrease in absorbance at 340 nm.

Preparation of DNA and transformation. Chromosomal DNA and plasmid DNA from C. glutamicum were isolated and purified as described by Follettie and Sinskey (1986). Plasmid DNA from E. coli was isolated by the method of Birnboim and Doly (1979) followed by CsCl density gradient. C. glutamicum was transformed via electroporation of whole cells as described by Liebl et al. (1989). E. coli was transformed by the CaCl₂ method of Cohen et al. (1973).

DNA manipulations. All restriction enzymes, T4 DNA ligase, Kle-noy polymerase, and calf intestine alkaline phosphatase were obtained from New England Biolabs (Schwalbach, FRG) or from Boehringer (Mannheim, FRG) and used as instructed by the manufacturer. Restriction-generated fragments were separated in 0.8% agarose gels and isolated and purified using the Geneclean™-Kit (Dianova, Hamburg, FRG) from BIO101.

### Table 1. Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype</th>
<th>Source or reference</th>
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<tbody>
<tr>
<td><strong>Corynebacterium glutamicum</strong></td>
<td></td>
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<tr>
<td>13032</td>
<td>Type strain</td>
<td>ATCC</td>
</tr>
<tr>
<td>DG 52-5</td>
<td>AEC⁸, aspartate kinase deregulated</td>
<td>Cremer et al. (1988), Degussa AG</td>
</tr>
<tr>
<td>DM 368-3</td>
<td>AEC⁸, AHV⁸, aspartate kinase and homoserine dehydrogenase deregulated</td>
<td>Degussa AG</td>
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<tr>
<td><strong>E. coli</strong></td>
<td></td>
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<tr>
<td>pEK0</td>
<td>C. glutamicum/E. coli shuttle vector: Km⁸, 6.3 kb</td>
<td>Kleintertz and Eikmanns, unpublished</td>
</tr>
</tbody>
</table>

Enzyme assays. For determination of enzyme activities cells were grown in 60 ml minimal medium to late exponential phase, washed twice in 20 ml of 50 mM TRIS/ HCl buffer, pH 7.6, containing 50 mM NaCl, and resuspended in 2 ml of 100 mM HEPES buffer, pH 7.6. The cells were disrupted by sonication with a microtip-equipped Branson (Danbury, Conn., USA) sonifier at maximal setting (2 × 2 min at 0°C). After centrifugation for 30 min at 13,000 g the supernatant was used for assays. The protein concentration of the extracts was determined by the method of Bradford (1976) using egg albumin as standard.

HDH (EC 1.1.1.3) was assayed as described by Follettie et al. (1988) at 30°C in 1 ml of 66 mM potassium phosphate buffer (pH 7.0), 0.25 mM NADPH, and 2.5 mM aspartic semialdehyde as substrate. The activity was determined by following the decrease in absorbance at 340 nm.

HK (EC 2.7.1.39) was assayed as described by Follettie et al. (1988) at 30°C in 1 ml of 100 mM HEPES buffer (pH 7.8) containing 250 mM KCl, 3.3 mM ATP, 0.25 mM NADH, 5 mM phosphoenolpyruvate, 10 mM MgCl₂, 10 units (U) pyruvate kinase (Boehringer), 25 U lactate dehydrogenase (Boehringer), and 5 mM L-homoserine as substrate. The activity was determined by following the decrease in absorbance at 340 nm.

TS (EC 4.2.99.2) activity was determined by the amount of inorganic phosphate released using a method described by Shames and Wedler (1984). The test was performed at 37°C for 30 min in 1 ml 20 mM HEPES buffer (pH 7.5) containing 200 mM KCl, 0.1 mM pyridoxal phosphate, 5 mM MgCl₂, 2 mM EDTA, 1 mM dithiothreitol, and 0.4 mM homoserine phosphate as substrate. The inorganic phosphate released was determined by the method of Lanzetta et al. (1979). Homoserine phosphate was prepared by the method of Skarstedt and Greer (1973) using cell free extracts of E. coli mutant gal 41.