Mol Gen Genet (1990) 224:317–324

MGG

Aspartokinase genes *lysCα* and *lysCβ* overlap and are adjacent to the aspartate β-semialdehyde dehydrogenase gene *asd* in *Corynebacterium glutamicum*

Jörn Kalinowski[1], Bernd Bachmann[2], Georg Thierbach[2] and Alfred Pühler[1]

[1] Lehrstuhl für Genetik, Fakultät für Biologie, Universität Bielefeld, Potfach 8640, W-4800 Bielefeld 1, Federal Republic of Germany
[2] DEGUSSA AG/ASTA Pharma AG, Kantstrasse 2, W-4802 Halle-Künsebeck, Federal Republic of Germany

Received March 2, 1990

Summary. A 2.1 kb DNA fragment of the recombinant plasmid pCS2, isolated from an aminoethyl cysteine (AEC)-resistant and lysine-producing *Corynebacterium glutamicum* mutant strain, and which confers AEC resistance and lysine production on the wild-type *G. glutamicum* ATCC 13032 was analysed. DNA sequence analysis of this fragment revealed three large open reading frames (ORFs). The incomplete ORF1 does not contain the 5′ end of the coding region. ORF2, which uses the same reading frame as ORF1, is identical to the 3′ end of ORF1 and encodes a putative protein of 172 amino acids (aa) and of M_r 18584. ORF3 encodes a putative protein of 344 aa and of M_r 36275. The amino acid sequences deduced from ORF1 and ORF2 display strong homologies to those of the α- and β-subunits of the *Bacillus subtilis* aspartokinase II. It is therefore proposed that the incomplete ORF1, termed *lysCα*, encodes part of the α-subunit of the *C. glutamicum* aspartokinase whereas the complete ORF2, termed *lysCβ*, encodes the β-subunit of the same enzyme. ORF2 is responsible for AEC resistance and lysine production due to a feedback-resistant aspartokinase. The amino acid sequence deduced from ORF3, termed *asd,* is highly homologous to that of the *Streptococcus mutans* aspartate β-semialdehyde dehydrogenase (ASD). Plasmids carrying the *C. glutamicum asd* gene complemented *Escherichia coli asd* mutants. Increase in ASD activity by a factor of 30–60 was measured for *C. glutamicum* cells harbouring high copy-number plasmids with the *C. glutamicum asd* gene.

Key words: Aminoethyl cysteine resistance – Aspartokinase – Aspartate β-semialdehyde dehydrogenase – DNA sequencing – Lysine production

Introduction

Corynebacteria are gram-positive non-sporulating bacteria. Several members of this taxonomic group are of special interest for industry, specifically for production of amino acids, steroid conversions, degradation of hydrocarbons and terpenoid oxidations (Martin et al. 1987). The most frequently used amino acid producers are *Brevibacterium flavum, Brevibacterium lactofermentum* and *Corynebacterium glutamicum.* Economically, L-lysine is one of the most important amino acids used mainly in forage addition (Tosaka et al. 1983).

The biosynthesis of lysine in amino acid-producing Corynebacteria is well understood and mainly follows the general scheme of L-lysine biosynthesis in bacteria via the diaminopimelic acid pathway (Tosaka and Takinami 1978). The main control of the so-called aspartate amino acid pathway branching to lysine, threonine, methionine and isoleucine is exerted via concerted inhibition of the aspartokinase enzyme by the end-products lysine plus threonine (Shiio and Miyajima 1969). Overproduction of lysine can be achieved by mutagenesis leading to strains with a deregulated aspartokinase enzyme which is no longer sensitive to feedback inhibition by a mixture of lysine and threonine (Shiio 1982). Such mutant strains can easily be selected by their resistance to a mixture of the L-lysine analogue S-(-2-aminoethyl)-D,L-cysteine (AEC) and L-threonine. *C. glutamicum* mutants showing a feedback-resistant aspartokinase (AK^{fbr}) excrete notable amounts of L-lysine (15–30 g/l).

In a recent publication (Thierbach et al. 1990), we described the cloning of a DNA fragment from an AEC-resistant and lysine-producing *C. glutamicum* mutant characterized by an aspartokinase activity that is completely insensitive to feedback inhibition. The cloned DNA fragment, carried by the recombinant plasmid pCS2, confers analogue resistance and lysine production to *C. glutamicum* wild-type strains. The aspartokinase activity of such a strain is partially feedback resistant. From these data it can be assumed that the cloned DNA fragment carries a deregulated aspartokinase gene.

In order to elucidate the origin of the AEC resistance carried by the recombinant plasmid pCS2, we determined the nucleotide sequence of the cloned *C. glutamicum* DNA and analysed the ORFs detected. It will be

Offprint requests to: Prof. Dr. A. Pühler

Table 1. Bacterial strains and plasmids used

Strain	Genotype relevant characteristics	Reference
Escherichia coli		
JM 83	*ara, Δ[lac, pro], strA, thi,* [∅80d*lacZ*ΔM15]	Messing 1979
RASA 51	*thi, asd*	Richaud et al. 1981
Corynebacterium glutamicum		
ATCC 13032	wild type, AEC^s	
DM58-1	AEC^r	Thierbach et al. 1990
Plasmids		
pSVB20	sequencing vector, Ap^r	Arnold and Pühler 1988
pSVB27	pSVB20 derivative with altered mcs	Arnold and Pühler 1988
pZ1	*E. coli-C. glutamicum* shuttle vector, Km^r and Ap^r	Menkel et al. 1989
pCS2	derivative of pZ1 with a 9.9 kb chromosomal DNA fragment, AEC^r	Thierbach et al. 1990
pCS26	*Sca*I deletion derivative of pCS2	Thierbach et al. 1990
pCS24	*Xho*I deletion derivative of pCS2	Thierbach et al. 1990
pCS23	*Sal*I deletion derivative of pCS2	Thierbach et al. 1990
pCS232	*Dra*I deletion derivative of pCS23	Thierbach et al. 1990
pCS233	*Eco*RI deletion derivative of pCS23	Thierbach et al. 1990

Abbreviations: AEC, S-(2-aminoethyl)-D,L-cysteine; Ap, ampicillin, Km, kanamycin; mcs, multiple cloning site

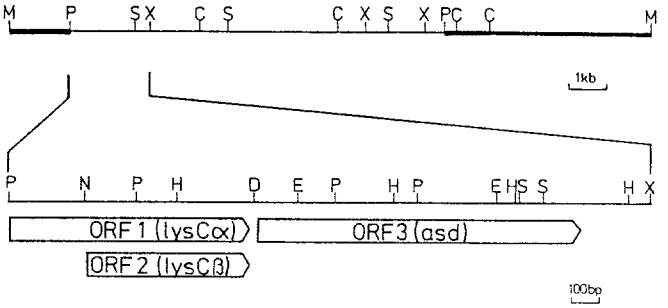

Fig. 1. Physical map and coding regions of a *Corynebacterium glutamicum PstI-XhoI* fragment responsible for AEC resistance and lysine production. The structure of the recombinant plasmid pCS2 containing the *Escherichia coli-C. glutamicum* shuttle vector (*thick line*) and a 9.9 kb *C. glutamicum* DNA fragment (*thin line*) as well as the physical map of a 2.1 kb *PstI-XhoI* subfragment are presented. Three open reading frames, later identified as *lysCα*, *lysCβ* and *asd* are shown as *open bars*. Abbreviations for restriction sites are as follows: C, *ScaI*; D, *DraI*; E, *Eco*RI; H, *Hin*dIII; M, *SmaI*; N, *NaeI*; P, *PstI*; S, *SalI*; X, *XhoI*

shown in this paper that the in-frame overlapping aspartokinase structural genes *lysCα* and *lysCβ* are clustered with the aspartate β-semialdehyde dehydrogenase gene *asd* and that the *lysCβ* gene is responsible for AEC resistance and lysine production.

Materials and methods

Bacterial strains and growth media. All bacterial strains and plasmids used are listed in Table 1. *E. coli* JM83 (Messing 1979) was used as recipient strain for cloning experiments. *E. coli* RASA 51 (Rickaud et al. 1981) was employed for complementation tests concerning the *C. glutamicum asd* gene. *E. coli* growth media were according to Miller (1972). They were supplemented with ampicillin (150 μg/ml) or kanamycin (50 μg/ml) or X-gal (40 μg/ml). *C. glutamicum* ATCC 13032 (wild type) and mutant strain DM58-1 (AEC^r) were grown in StI or minimal medium according to Thierbach et al. (1988). Cells harbouring recombinant plasmids were selected on agar plates supplemented with kanamycin (25 μg/ml and 10 μg/ml, respectively), D,L-AEC (10 mg/ml) and L-threonine (2 mg/ml).

Molecular cloning and sequencing procedures. DNA restriction and separation by gel electrophoresis, DNA ligation and transformation of *E. coli* were performed by standard methods described by Maniatis et al. (1982). Plasmid DNA of *E. coli* was prepared essentially as described by Holmes and Quigley (1981). Nucleotide sequences were determined by the chemical degradation method (Maxam and Gilbert 1977) with the modifications introduced by Barker et al. (1983). DNA fragments were cloned into the pUC18 plasmid derivatives pSVB20 and pSVB27 (Arnold and Pühler 1988) and single-ends were labelled using the unique *Bst*EII restriction site of these vectors. Sequencing gels were prepared as described by Garoff and Ansorge (1981). The DNA sequence was analysed with the sequence analysis programs of Staden (1986) and Lipman and Pearson (1985).

Restriction enzymes and T4 DNA ligase were purchased from BRL, Karlsruhe, FRG and Klenow fragment of *E. coli* DNA Polymerase from Boehringer, Mannheim, FRG. [α-^{32}P]dCTP was from NEN, Dreieich, FRG.

Preparation of crude protein extracts of C. glutamicum *and assay for aspartate β-semialdehyde dehydrogenase activity*

Growth conditions of *C. glutamicum* strains and preparation of crude protein extracts were as described by Thierbach et al. (1990). The aspartate β-semialdehyde dehydrogenase assay used the following reaction: 120 mM diethanolamine (pH 9.0), 40 mM $NaAsO_2$, 1 mM $NADP^+$, 5 mM L-threonine, 1.3 mM aspartate β-semialdehyde and different amounts of crude extracts were mixed in a total volume of 1 ml. The enzyme activity was determined by the velocity of NADPH synthesis (ΔA_{340}) and given as μmol/mg protein/min (U/mg). Protein concentrations were measured by the methods of Bradford (1976) and Lowry et al. (1951).

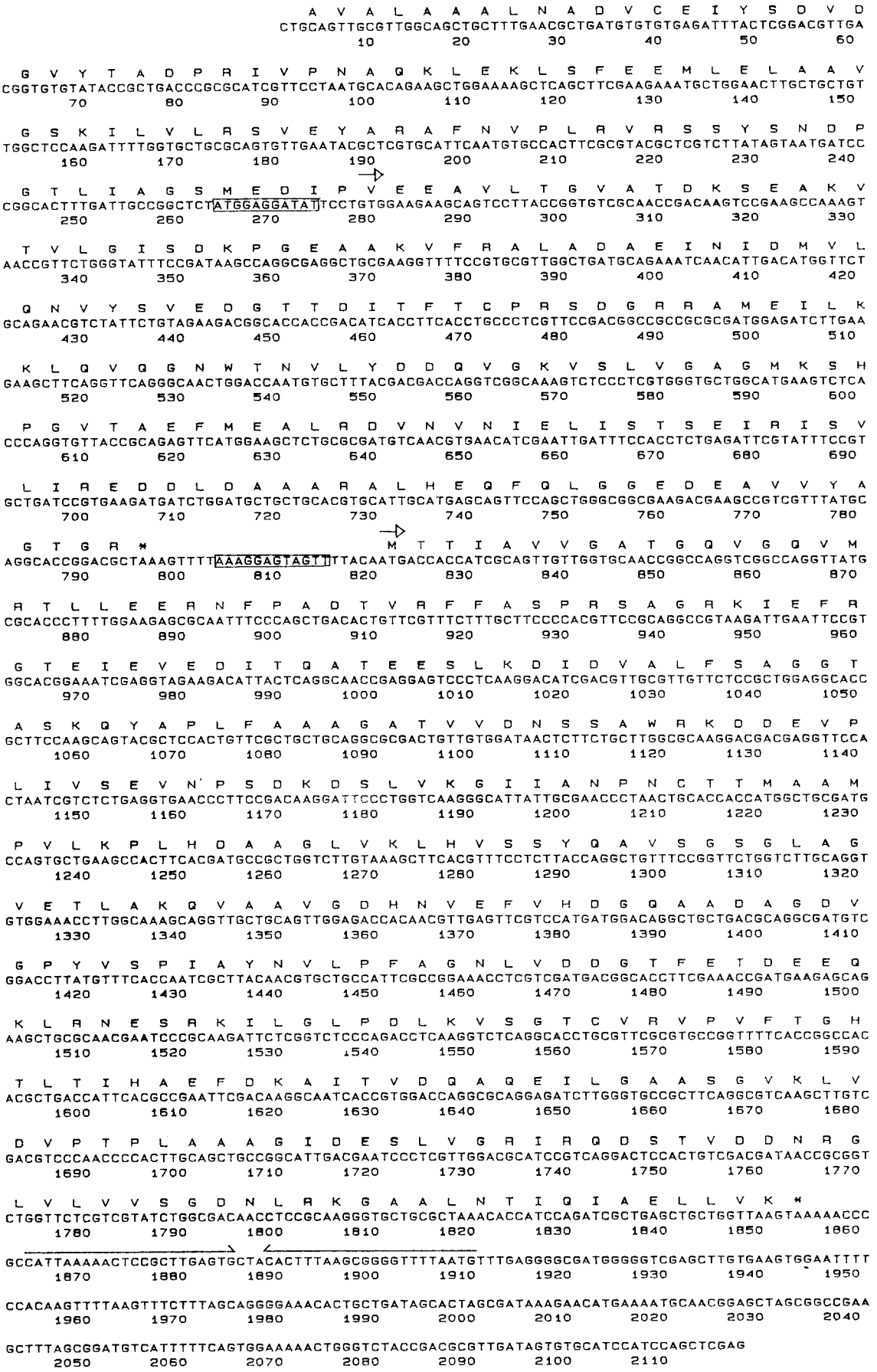

Fig. 2. Nucleotide sequence of the *C. glutamicum* *Pst*I-*Xho*I fragment responsible for AEC resistance and lysine production. The nucleotide sequence of one strand of the DNA fragment is presented in 5′ to 3′ direction. The amino acid sequences of the identified open reading frames are also shown. Putative ribosomal binding sites are *boxed*. The assumed start codons of the open reading frames are indicated by *short arrows* above the amino acid sequence. *Bold-lined arrows* above the nucleotide sequence indicate a possible ρ-independent transcription terminator

Results

A 2.1 kb C. glutamicum *DNA fragment conferring AEC resistance and lysine production carries three open reading frames*

The recombinant plasmid pCS2 consisting of the *E. coli-C. glutamicum* shuttle vector pZ1 and a 9.9 kb *C. glutamicum* DNA fragment was shown to contain a 2.1 kb *Pst*I-*Xho*I fragment (Fig. 1), which was responsible for AEC resistance and lysine production (Thierbach et al. 1990). The nucleotide sequence of this fragment was determined by the chemical degradation method (Maxam and Gilbert 1977). The sequence obtained, comprising 2118 bp, is presented in Fig. 2. With the help of the computer programs of Staden (1986) a coding region analysis was carried out. As a result, three large open reading frames (ORF) with high coding probability were identified on one DNA strand (Fig. 1).

The first open reading frame, ORF1, later identified as part of *lys*Cα, is located between nucleotides 1 and 794. ORF1 is obviously incomplete since it does not contain an ATG or a GTG start codon preceded by an appropriate ribosome-binding site. The truncated ORF1 codes for 264 amino acids. The second open reading frame ORF2, later identified as *lys*Cβ, extends from nucleotides 279 to 794 and uses the same reading frame as ORF1. Therefore ORF2 is identical to the 3′ end of ORF1. ORF2 contains a GTG start codon and is preceded by the putative ribosome-binding site (RBS) 5′-ATGGAGGATAT-3′ located between nucleotides 264 and 274. This RBS sequence fits the sequence of the 3′ end of the *Bacillus subtilis* 16S rRNA (Moran et al. 1982) in 8 out of 11 positions (underlined). ORF2 encodes a protein of 172 amino acids and with a molecular mass of 18584. The third open reading frame ORF3, later identified as *asd,* is located between nucleotides 821 and 1852. It starts with an ATG that is preceded by the putative RBS sequence 5′-AAAGGAGTAGTT-3′ (nucleotides 804–815) where 9 out of 12 nucleotides match the 3′ end of the *B. subtilis* 16S rRNA (underlined). ORF3 encodes a protein of 344 amino acids and with a molecular mass of 36275.

The identified ORFs are separated by only a short intergenic region of 23 nucleotides, which is too short to carry terminator or promoter structures. A DNA sequence of 48 nucleotides representing an inverted repeat is located immediately behind ORF3 (Fig. 2). It strongly resembles a ρ-independent *E. coli* transcription terminator (Adhya and Gottesman 1978). The most stable conformation of the hairpin loop formed by this inverted repeat has a $\Delta G = -76.0$ kJoule/mol, as calculated by the rules of Tinoco et al. (1973).

The amino acid sequences deduced from ORF1 and ORF2 are homologous to the amino acid sequence of the B. subtilis *aspartokinase*

The FASTA program package (Lipman and Pearson 1985) was used to compare the amino acid sequence deduced from ORF1 to the amino acid sequences stored in the NBRF Protein Sequence Database. A high degree of homology was found between the amino acid sequence of the *B. subtilis* aspartokinase II (Chen et al. 1987) and the amino acids sequence of ORF1 (Fig. 3). The region of homology begins with amino acid (aa) 1 of the sequence deduced from ORF1 and with aa 157 of the *B. subtilis* aspartokinase II. Altogether a homology of 38% was calculated between the amino acid sequences deduced from ORF1 and the corresponding part of the *B. subtilis* aspartokinase II. These results indicate that the incomplete ORF1 represents a truncated aspartokinase gene devoid of its 5′ end. The amino acid sequence of ORF1 also shows significant homologies to the different *E. coli* aspartokinase gene products. In addition, it was found that the *E. coli* aspartokinase I-homoserine dehydrogenase I (Katinka et al. 1980)

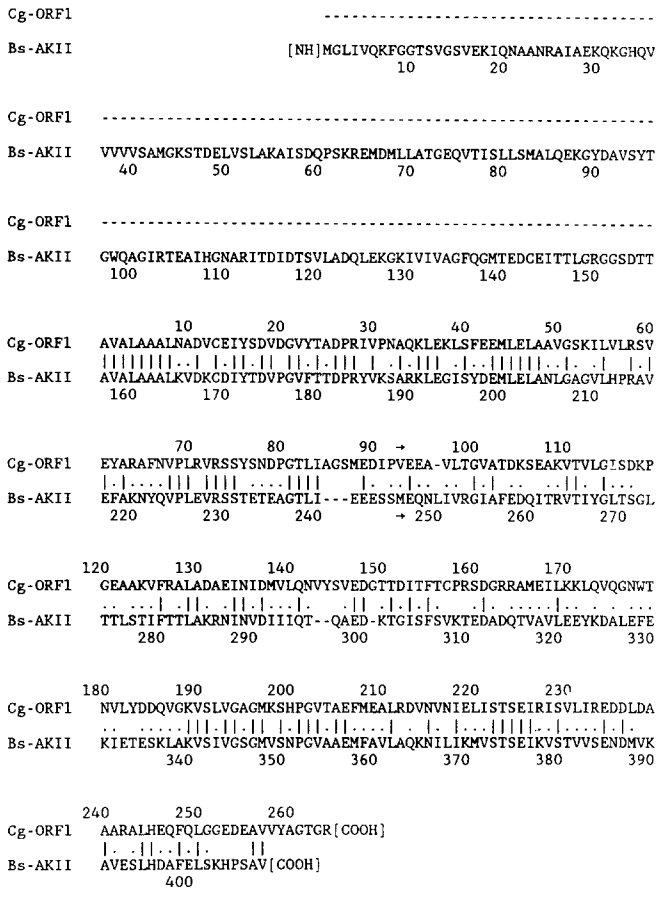

Fig. 3. Alignment of the amino acid sequences deduced from *C. glutamicum* ORF1 and ORF2 with that of the *Bacillus subtilis* aspartokinase (Chen et al. 1987). The amino acid sequences were aligned with the aid of the FASTA computer program (Lipman and Pearson 1985). The alignment of the sequence from *C. glutamicum* starts with the first residue encoded by the cloned fragment. Identical residues are marked by *vertical lines*. Similar residues as identified by means of the Dayhoff matrix (Dayhoff et al. 1983), are marked by *dots*. *Hyphens* indicate missing N-terminal amino acids that are not encoded by the incomplete ORF1. The start codons of the β-subunits (ORF1 of *C. glutamicum*) are shown by *arrows*.
Abbreviations: Cg-ORF1, *C. glutamicum* ORF1; Bs-AKII, *B. subtilis* aspartokinase II

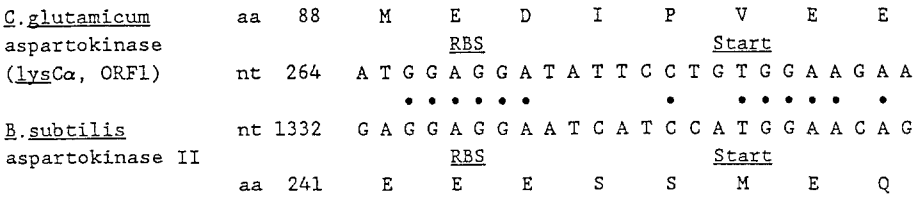

Fig. 4. Alignment of the nucleotide sequences around the start site of *C. glutamicum* aspartokinase (ORF2) and the second translational start site in the *B. subtilis* aspartokinase II gene. The deduced amino acid sequences are also shown. The first nucleotides (nt) and amino acids (aa) of this section are indicated. Identical nucleotides are marked by *solid dots* and the ribosome-binding sites (RBS) and the start codons (Start) are indicated

shows 30% homology, the aspartokinase II-homoserine dehydrogenase II (Zakin et al. 1983) 23% homology and the aspartokinase III (Cassan et al. 1986) 26% homology when compared to the truncated *C. glutamicum* aspartokinase encoded by ORF1.

Since ORF1 encodes a protein with homology to *B. subtilis* and *E. coli* aspartokinases we propose to term the incomplete *C. glutamicum* ORF1 a truncated *lys*C gene. The *lys*C gene was first described in *E. coli* to code for aspartokinase III which is regulated by lysine and not fused with a homoserine dehydrogenase domain (Thèze et al. 1974). Also in *C. glutamicum* lysine is one of the feedback inhibitors of the aspartokinase and ORF1 evidently does not encode a homoserine dehydrogenase part.

Since ORF2 is part of the 3′ end of ORF1 and since ORF2 uses the same reading frame as ORF1 the amino acid sequence deduced from ORF2 is also homologous to the *B. subtilis* aspartokinase II (Fig. 3). The calculated degree of homology is 28%. It is of interest that for the *B. subtilis* aspartokinase II gene a second translational start was postulated at amino acid position 246 (Chen et al. 1987; Fig. 4). This second translational start corresponds to that of the *C. glutamicum* ORF2; ribosome-binding sites and start codons are located at identical positions. In this region, the homology of the amino acid sequences is low but the degree of the nucleotide sequence similarity is rather high, implicating conserved signals at the DNA level.

Taking into account the sequence information obtained and also the structural model for the *B. subtilis* aspartokinase containing two different subunits (Moir and Paulus 1977b) we propose that in *C. glutamicum* two genes, ORF1 and ORF2, code for the subunits of the aspartokinase. The incomplete ORF1 encodes part of the α-subunit, whereas ORF2 encodes the β-subunit of the aspartokinase holoenzyme. According to the above designation these in-frame overlapping genes were termed *lys*Cα and *lys*Cβ.

The amino acid sequence deduced from ORF3 is homologous to the amino acid sequence of the Streptococcus mutans *aspartate β-semialdehyde dehydrogenase (ASD)*

The amino acid sequence of ORF3 was compared to the amino acid sequences stored in the NBRF protein database using again the FASTA programs (Lipman and Pearson 1985). The amino acid sequence of the *Streptococcus mutans* aspartate β-semialdehyde dehydrogenase (Cardineau and Curtiss 1987) was found to be homologous to the sequence derived from ORF3 (Fig. 5). Altogether a homology of 39% between the two amino acid sequences was calculated.

Based on this sequence information we designated the *C. glutamicum* ORF3 an *asd* gene. Other proteins that display homology to the product of ORF3 are the *E. coli* ASD (Haziza et al. 1982) and the glyceraldehyde-3-phosphate dehydrogenase proteins (GAPDH) of *E. coli* (Branlant and Branlant 1985), *B. subtilis* (Viaene and Dhaese 1989) and *Bacillus stearothermophilus* (Branlant et al. 1989).

Functional analysis of the C. glutamicum asd *and* lysCβ *genes cloned on plasmid pCS2*

To analyse the function of the *C. glutamicum asd* gene located on plasmid pCS2 complementation tests with an *E. coli asd*$^-$ strain were carried out. For this purpose we took advantage of several derivatives of pCS2 (Thierbach et al. 1990) with deletions in the inserted *C. glutamicum* chromosomal DNA (Fig. 6). The plasmids pCS2, pCS26 and pCS24 all contain *lys*Cβ as well as *asd*, whereas in pCS23, pCS232 and pCS233 the *asd* gene is incomplete. As expected, only the plasmids pCS2, pCS26 and pCS24 were able to complement the *E. coli asd* mutant (Fig. 6), demonstrating that the identified *C. glutamicum* ORF3 encodes an aspartate β-semialdehyde dehydrogenase.

Plasmid pCS2 and all of its derivatives with an intact *lys*Cβ conferred AEC resistance and lysine production on the *C. glutamicum* wild type (Fig. 6). The nearly complete removal of the *asd* coding sequence in plasmid pCS233 does not abolish this phenotype but the exact deletion of ORF1 and ORF2 (pCS232) causes AEC sensitivity and prevents lysine production. From these results it can be concluded that ORF2 (*lys*Cβ) alone is responsible for AEC resistance and lysine production because ORF1 as a truncated *lys*Cα gene without promoter and translational start site is obviously not expressed.

In order to quantify the effect of *asd*-containing plasmids in *C. glutamicum*, crude extracts of *C. glutamicum* strains carrying the different recombinant plasmids of Fig. 6 were assayed for aspartate β-semialdehyde dehydrogenase (ASD) enzyme activity. All the plasmids car-

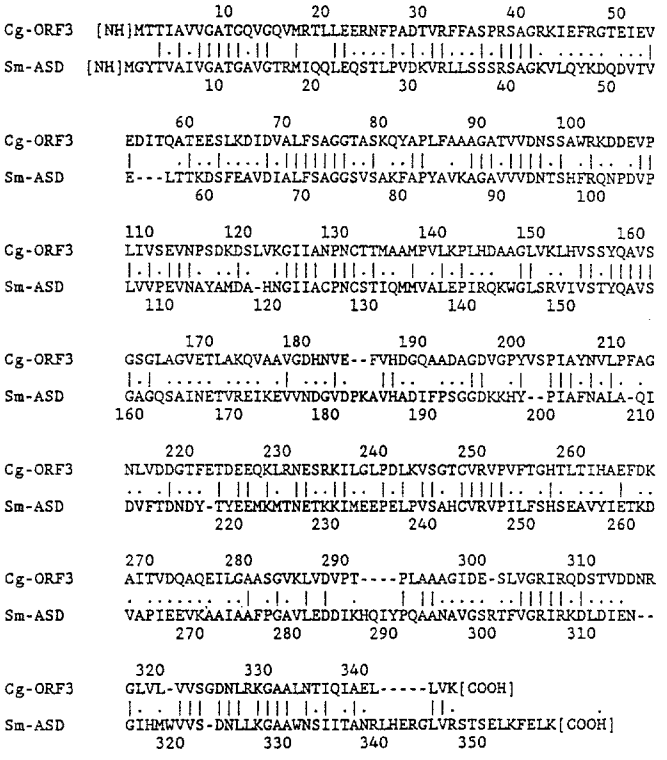

Fig. 5. Alignment of the amino acid sequences deduced from *C. glutamicum* ORF3 (Cg-ORF3) with the amino acid sequence of the *Streptococcus mutans* aspartate-semialdehyde dehydrogenase (Sm-ASD) (Cardineau and Curtiss 1987). Identical residues are shown by *vertical lines*. Similar residues, as identified by means of the Dayhoff matrix (Dayhoff et al. 1983), are marked by *dots*

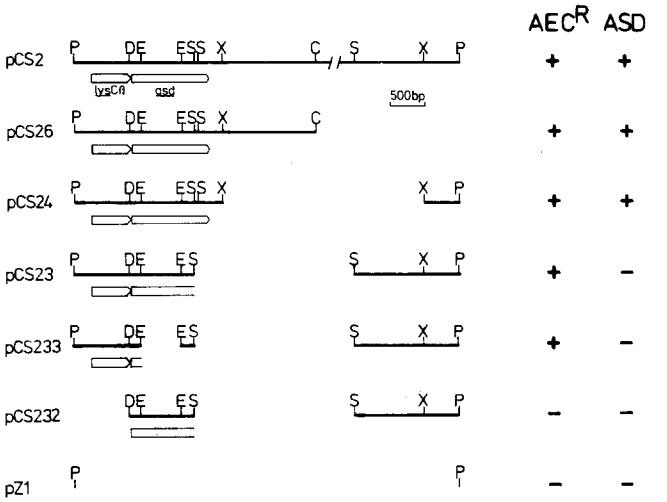

Fig. 6. Functional analysis of deletion derivatives of plasmid pCS2 in AEC-sensitive *C. glutamicum* and ASD-defective *E. coli* strains. The physical maps of the inserts of *C. glutamicum* DNA cloned in pCS2 and its deletion derivatives are presented. The translated DNA regions of *lysCβ* and *asd* are shown below the physical maps. On the right of the figure the phenotypes mediated by the plasmids are listed. AEC^R: resistance of plasmid carrying *C. glutamicum* cells against mixtures of 50 mM DL-AEC and 50 mM L-threonine. ASD: growth of plasmid carrying *E. coli* RASA 51 (*asd*$^-$; Richaud et al. 1981) on LB agar without addition of diaminopimelic acid. Abbreviations for restriction endonuclease sites are as given for Fig. 1; only the relevant sites for *Dra*I, *Eco*RI and *Pst*I of the insert are shown

Table 2. Aspartate β-semialdehyde dehydrogenase (ASD) activity of *C. glutamicum* strains carrying pZ1, pCS2 or different deletion derivatives of plasmid pCS2

Strain	Plasmid	ASD (U/mg)
ATCC 13032	pZ1	0.06
DM58-1	pZ1	0.33
ATCC 13032	pCS2	3.90
ATCC 13032	pCS26	1.88
ATCC 13032	pCS24	2.07
ATCC 13032	pCS23	0.03

rying an intact *asd* gene mediate a remarkable increase in specific ASD activity to *C. glutamicum* (Table 2). The amplification is 30- to 60-fold relative to the activity of the wild-type *C. glutamicum* ATCC 13032 carrying the plasmid vector pZ1 and 6- to 12-fold with respect to mutant strain *C. glutamicum* DM58-1 (pZ1). DM58-1 is a lysine-excreting mutant which was used for cloning the *lysCβ* and the *asd* gene of plasmid pCS2. The amplification rate evidenced in ASD activity is due to the gene dosage effect caused by the high copy number of the recombinant plasmids.

Discussion

Organisation of the C. glutamicum *genes* lysCα, lysCβ *and* asd

In this paper we showed that the overlapping aspartokinase genes *lysCα* and *lysCβ* and the aspartate β-semialdehyde dehydrogenase gene *asd* are clustered in *C. glutamicum*. This result was achieved by sequencing the 2.1 kb DNA insert of plasmid pCS2 responsible for lysine production and AEC resistance (Thierbach et al. 1990). The *lysCα* gene is incomplete on pCS2. Its 5′ end is missing but it contains the complete *lysCβ* gene which is identical to the 3′ end of *lysCα*. The *asd* gene is separated from *lysCα*/*lysCβ* by a small intergenic region and delimited by a hairpin loop structure probably representing a transcriptional terminator.

This arrangement strongly resembles a biosynthetic operon. However, the promoter of this putative operon is not yet known. From DNA sequence data of the cloning vector pZ1 there are no indications for a transcriptional fusion of a vector promoter with the *lysCβ* and the *asd* gene. On the other hand, upstream of *lysCβ* and within the *lysCα* coding region there are several *E. coli*-promoterlike DNA sequences which could act as transcriptional starts (data not shown). A detailed analysis is necessary to characterize the promoter arrangement in the putative *lysC-asd* operon of *C. glutamicum*.

Subunit structure and conformation of the C. glutamicum *aspartokinase*

The *C. glutamicum lysCα* and *lysCβ* genes were identified by comparing the derived amino acid sequences to those

of the *B. subtilis* aspartokinase II α- and β-subunits. In addition, all three aspartokinases of *E. coli* exhibit a remarkable degree of similarity to the α-subunit of the *C. glutamicum* aspartokinase (data not shown). The identified aspartokinase genes of *C. glutamicum* were termed *lys*Cα and *lys*Cβ. The incomplete *lys*Cα gene present on plasmid pCS2 is devoid of most of the DNA region coding for the N-terminal domain, which catalyses aspartylphosphate formation as shown by limited proteolysis of the *E. coli* aspartokinases (Fazel et al. 1983).

In *B. subtilis* the β-subunit of aspartokinase is the result of an independent translational start at a strong ribosome-binding site within the coding region of the α-subunit (Bondaryk and Paulus 1985; Chen and Paulus 1988). Neither catalytic nor regulatory functions could be assigned to the *B. subtilis* aspartokinase β-subunit (Chen and Paulus 1988), but it was found that α- and β-subunits are present in the native aspartokinase in equimolar amounts. Furthermore, the catalytically active form of *B. subtilis* aspartokinase represents a tetramer of the conformation $\alpha_2\beta_2$ (Moir and Paulus 1977a). According to the homologies identified between the aspartokinase genes from *B. subtilis* and *C. glutamicum* it seems reasonable to propose a similar conformation for the *C. glutamicum* aspartokinase.

Mediation of AEC resistance by a mutated lys*Cβ gene* (lys$C\beta^{fbr}$)

In this paper we demonstrated that the *lys*Cβ gene isolated from a *C. glutamicum* mutant with a feedback-resistant aspartokinase (AK^{fbr}) is responsible for AEC resistance. In addition, it is known that a *C. glutamicum* wild-type strain harbouring plasmids with the same *lys*Cβ gene shows a partially feedback-resistant aspartokinase activity and produces lysine (Thierbach et al. 1990). From these results it can be concluded that the cloned *lys*Cβ gene described here is actually a mutated *lys*Cβ which is responsible for the feedback-resistant aspartokinase. For this reason we would like to name the cloned *lys*Cβ gene *lys*$C\beta^{fbr}$. Of course, this conclusion has to be confirmed by cloning the wild-type *lys*Cβ and by identifying the mutation leading to *lys*$C\beta^{fbr}$.

The partially deregulated aspartokinase enzyme isolated from a *C. glutamicum* wild-type strain harbouring a plasmid with *lys*$C\beta^{fbr}$ can be logically explained with the assumed $\alpha_2\beta_2$ conformation of the aspartokinase. Since the strain mentioned above also carries a chromosomal *lys*Cβ gene, two types of subunits should be produced: β^{fbr} and β^{wt}. This means that in such a strain a mixture of aspartokinases differing in their degree of feedback inhibition should be formed. The following combinations are possible: $\alpha_2\beta_2{}^{wt}$, $\alpha_2\beta^{wt}\beta^{fbr}$ and $\alpha_2\beta_2{}^{fbr}$. It is clear that such a mixture is only partially feedback resistant as indicated in our previous paper (Thierbach et al. 1990). Additionally, it has to be considered that a mutation in the *lys*Cβ gene is also expressed in the product derived from the *lys*Cα gene in a strain having the mutated aspartokinase genes in the chromosome. It is therefore possible that only the aspartokinase $\alpha_2{}^{fbr}\beta_2{}^{fbr}$ is completely resistant to feedback inhibition.

C. glutamicum *aspartate β-semialdehyde dehydrogenase*

The *C. glutamicum asd* gene coding for the aspartate β-semialdehyde dehydrogenase located on plasmid pCS2 was identified by homology studies of its derived amino acid sequence and by complementation of an *E. coli* mutant (Fig. 6). The amino acid sequence of the *C. glutamicum asd* gene product is in good agreement with the amino acid sequence of the aspartate β-semialdehyde dehydrogenase from the gram-positive bacterium *Streptococcus mutans* (Fig. 5).

The levels of ASD enzyme activity are increased 30- to 60-fold in *C. glutamicum* wild-type strains carrying multicopy plasmids with the genes *lys*$C\beta^{fbr}$ and *asd*. The increase can be explained by the copy number of the plasmids but it should be mentioned that the original strain DM58-1 characterized by a feedback-resistant aspartokinase itself exhibits a 5-fold higher level of enzymatic activity when compared to a wild-type *C. glutamicum* strain (Table 2). The reason for *asd* overexpression in strain DM58-1 is not clear and remains to be investigated.

Acknowledgements. This work was financed by grant 038409 from the Bundesministerium für Forschung und Technologie. Additional financial support was provided by the Fonds der chemischen Industrie. We wish to thank M. Rustemeier, S. Sieben and C. Skroblin for excellent technical assistance.

References

Adhya S, Gottesman M (1978) Control of transcription termination. Annu Rev Biochem 47:967–996

Arnold W, Pühler A (1988) A family of high-copy-number plasmid vectors with single-end-label sites for rapid nucleotide sequencing. Gene 70:171–179

Barker RF, Idler KB, Thompson DV, Kemp JD (1983) Nucleotide sequence of T-DNA region from the *Agrobacterium tumefaciens* octopine Ti plasmid pTi15955. Plant Mol Biol 2:335–350

Bondaryk RP, Paulus H (1985) Expression of the gene for *Bacillus subtilis* aspartokinase II in *Escherichia coli*. J Biol Chem 260:592–597

Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein dye binding. Anal Biochem 72:248–254

Branlant G, Branlant C (1985) Nucleotide sequence of the *Escherichia coli gap* gene. Eur J Biochem 150:61–66

Branlant C, Oster T, Branlant G (1989) Nucleotide sequence determination of the DNA region coding for *Bacillus stearothermophilus* glyceraldehyde-3-phosphate dehydrogenase and of the flanking DNA regions required for its expression in *Escherichia coli*. Gene 75:145–155

Cardineau GA, Curtis III R (1987) Nucleotide sequence of the *asd* gene of *Streptococcus mutans*. J Biol Chem 262:3344–3353

Cassan M, Parsot C, Cohen GN, Patte J-C (1986) Nucleotide sequence of *lys*C gene encoding the lysine-sensitive aspartokinase III of *Escherichia coli* K12. J Biol Chem 261:1052–1057

Chen N-Y, Paulus H (1988) Mechanism of expression of the overlapping genes of *Bacillus subtilis* aspartokinase II. J Biol Chem 263:9526–9532

Chen N-Y, Hu F-M, Paulus H (1987) Nucleotide sequence of the overlapping genes for the subunits of *Bacillus subtilis* aspartokinase II and their control regions. J Biol Chem 262:8787–8798

Dayhoff MO, Barker WC, Hunt LT (1983) Establishing homologies in protein sequences. Methods Enzymol 91:524–545

Fazel A, Guillou Y, Cohen GN (1983) A hybrid proteolytic fragment of *Escherichia coli* aspartokinase I-homoserine dehydrogenase I. J Biol Chem 258:13570–13574

Garoff H, Ansorge W (1981) Improvement of DNA sequencing gels. Anal Biochem 115:450–457

Haziza C, Stragier P, Patte J-C (1982) Nucleotide sequence of the *asd* gene of *Escherichia coli:* absence of a typical attenuation signal. EMBO J 1:379–384

Holmes DS, Quigley M (1981) A rapid boiling method for the preparation of bacterial plasmids. Anal Biochem 114:193–197

Katinka M, Cossart P, Sibilli L, Saint-Girons I, Chelvignac MA, LeBras G, Cohen GN, Yaniv M (1980) Nucleotide sequence of the *thr*A gene of *Escherichia coli.* Proc Natl Acad Sci USA 77:5730–5733

Lipman DJ, Pearson WR (1985) Rapid and sensitive protein similarity searches. Science 227:1435–1441

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the folin phenol reagent. J Biol Chem 193:265–275

Maniatis T, Fritsch EF, Sambrook J (1982) Molecular cloning: A laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York

Martin JF, Santamaria R, Sandoval H, del Real G, Mateos LM, Gil JA, Aguilar A (1987) Cloning systems in amino acid-producing Corynebacteria. Bio/Technology 5:137–146

Maxam AM, Gilbert W (1977) A new method for sequencing DNA. Proc Natl Acad Sci USA 74:560–564

Menkel E, Thierbach G, Eggeling L, Sahm H (1989) Influence of increased aspartate availability on lysine formation by a recombinant strain of *Corynebacterium glutamicum* and utilization of fumarate. Appl Environ Microbiol 55:684–688

Messing J (1979) A multi-purpose cloning system based on the single-stranded DNA bacteriophage M13. Recombinant DNA Technical Bulletin, NIH Publication No. 79–99 2:43–48

Miller JH (1972) Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York

Moir D, Paulus H (1977a) Properties and subunit structure of aspartokinase II from *Bacillus subtilis* VB217. J Biol Chem 252:4648–4654

Moir D, Paulus H (1977b) Immunological and chemical comparison of the nonidentical subunits of aspartokinase II from *Bacillus subtilis* VB217. J Biol Chem 252:4655–4661

Moran CP Jr, Lang N, LeGrice SFJ, Lee G, Stephens M, Sonenshein AL, Pero J, Losick R (1982) Nucleotide sequences that signal the initiation of transcription and translation in *Bacillus subtilis.* Mol Gen Genet 186:339–346

Richaud F, Richaud C, Haziza C, Patte J-C (1981) Isolement et purification de gênes d'*Escherichia coli* K12 impliqués dans la biosynthèse de la lysine. C R Acad Sci Paris 293:507–512

Shiio I (1982) Metabolic regulation and over-production of amino acids. In: Krumphanzl V, Sikyta B, Vanek Z (eds) Overproduction of microbial products. Academic Press, London, pp 463–472

Shiio I, Miyajima R (1969) Concerted inhibition and its reversal by end products of aspartate kinase in *Brevibacterium flavum.* J Biochem 65:849–859

Staden R (1986) The current status and portability of our sequence handling software. Nucleic Acids Res 14:217–232

Thèze J, Margarita D, Cohen GN, Borne F, Patte JC (1974) Mapping of the structural genes of the three aspartokinases and of the two homoserine dehydrogenases of *Escherichia coli* K12. J Bacteriol 117:133–143

Thierbach G, Schwarzer A, Pühler A (1988) Transformation of spheroplasts and protoplasts of *Corynebacterium glutamicum.* Appl Microbiol Biotechnol 29:356–362

Thierbach G, Kalinowski J, Bachmann B, Pühler A (1990) Cloning of a DNA fragment from *Corynebacterium glutamicum* conferring aminoethyl cysteine resistance and feedback resistance to aspartokinase. Appl Microbiol Biotechnol 32:443–448

Tinoco I Jr, Borer BN, Dengler B, Levine MD, Uhlenbeck OC, Crothers DM, Gralla J (1973) Improved estimation of secondary structure in ribonucleic acid. Nature New Biology 246:40–41

Tosaka O, Takinami K (1978) Pathway and regulation of lysine biosynthesis in *Brevibacterium lactofermentum.* Agric Biol Chem 42:95–100

Tosaka O, Enei H, Hirose Y (1983) The production of L-lysine by fermentation. Trends Biotechnol 1:70–76

Viaene A, Dhaese P (1989) Sequence of the glyceraldehyde-3-phosphate dehydrogenase gene from *Bacillus subtilis.* Nucleic Acids Res 17:1251

Zakin MM, Duchange N, Ferrara P, Cohen GN (1983) Nucleotide sequence of the *met*L gene of *Escherichia coli:* its product, the bifunctional aspartokinase II-homoserine dehydrogenase II, and the bifunctional product of the *thr*A gene, aspartokinase I-homoserine dehydrogenase I derive from a common ancestor. J Biol Chem 258:3028–3031

Communicated by C.P. Hollenberg