Genes and enzymes of the acetyl cycle of arginine biosynthesis in *Corynebacterium glutamicum*: enzyme evolution in the early steps of the arginine pathway

Vehary Sakanyan,¹ Pavel Petrosyan,² Michèle Lecocq,¹ Anne Boyen,^{3,4} Christiane Legrain,⁵ Marc Demarez,⁵ Jean-Noël Hallet¹ and Nicolas Glansdorff^{3,4,5}

Author for correspondence: Vehary Sakanyan. Tel: +33 40373065. Fax: +33 40292042.

¹ Laboratoire de Biotechnologie, Faculté des Sciences et des Techniques, Université de Nantes, 2, rue de la Houssinière, F-44072 Nantes Cedex 03, France

² Pharmagen, Knunyants Street 4, Yerevan 375010, Republic of Armenia

^{3,4,5} Microbiologie, Vrije Universiteit Brussel³, Vlaams Interuniversitair Instituut voor Biotechnologie⁴, Research Institute, CERIA-COOVI⁵, 1 avenue Emile Gryson, B-1070 Brussels, Belgium

A cluster of arginine biosynthetic genes of *Corynebacterium glutamicum* ATCC 13032, comprising *argJ*, *argB* and *argD* as well as part of *argC* and *argF*, has been cloned by heterologous complementation of an *Escherichia coli* *argE* mutant. The gene order has been established as *argCJBDF* by sequencing the entire 4.4 kb cloned DNA fragment. The *C. glutamicum* *argB* gene can be transcribed in *E. coli* cells from an internal promoter located in the coding part of the preceding *argJ* gene, whereas transcription of the *argJ* gene appears vector-dependent. Expression of the corynebacterial *argB* gene is repressed by arginine in the native host but not in recombinant *E. coli* cells. Feedback inhibition of the corresponding *N*-acetylglutamate kinase activity was observed both in cell extracts of *C. glutamicum* and in recombinant *E. coli* *argB* auxotrophic strains. Extracts of *E. coli* cells carrying cloned corynebacterial DNA display an ornithine acetyltransferase activity (encoded by *argJ*) which alleviates the acetylornithinase (encoded by *argE*) deficiency of the enterobacterial host. In contrast to *Bacillus stearothermophilus* ornithine acetyltransferase which also exhibits acetylglutamate synthase activity, *C. glutamicum* ornithine acetyltransferase appears monofunctional. ArgA and ArgB proteins from different sources share highly significant similarities. The evolutionary implications of these data are discussed.

Keywords: *Corynebacterium glutamicum*, *argCJBDF* sequence, ornithine acetyltransferase, *N*-acetylglutamate kinase, regulation

INTRODUCTION

Arginine biosynthesis sets off with the acetylation of the amino group of glutamate (Fig.1), mediated by *N*-acetylglutamate synthase (acetylCoA:L-glutamate *N*-acetyltransferase; EC 2.3.1.1; the *argA* gene product). This acetylation prevents spontaneous cyclization and hence proline formation during the subsequent modifications of the 5-carboxyl group. Two strategies have evolved for the ulterior removal of the acetyl group. In the so-called linear pathway operative in *Enterobacteriaceae* and in the archaeon *Sulfolobus solfataricus* acetylornithinase (*N*²-acetyl-L-ornithine amidohydrolase; EC 3.5.1.16; the *argE* gene product) catalyses the hydrolysis of *N*-

acetylornithine into the arginine precursor ornithine and acetate (Cunin *et al.*, 1986; Van de Casteele *et al.*, 1990). All other prokaryotes investigated up to now, including methanogenic archaea (Meile & Leisinger, 1984; Van de Casteele *et al.*, 1990), *Neisseria gonorrhoeae* (Shinners & Catlin, 1978), members of the genus *Bacillus* (Sakanyan *et al.*, 1992) and the eukaryotic microbes (Davis, 1986) use the more economical cyclic pathway that was first brought to light in *Micrococcus glutamicus* (Udaka & Kinoshita, 1958), in which the acetyl group is recycled with generation of acetylglutamate. The transacetylation between acetylornithine and glutamate is mediated by the *argJ* gene product, ornithine acetyltransferase (*N*²-acetyl-L-ornithine:L-glutamate *N*-acetyltransferase; EC 2.3.1.35). In organisms depending on the cyclic pathway, *N*-acetylglutamate synthase therefore fulfills an anaplerotic function.

The EMBL accession number for the sequence reported in this paper is X86157.

Fig. 1. Arginine biosynthesis and its relationship to the proline pathway. The dashed line indicates the acetyl group recycling by the *argJ*-encoded enzyme as an alternative to deacetylation by the *argE* gene product.

Complementation experiments with *N. gonorrhoeae* (Picard & Dillon, 1989; Martin & Mulks, 1992) and *Bacillus stearothermophilus* (Sakanyan *et al.*, 1990) revealed that one small DNA fragment of these organisms could complement both *argA* and *argE* auxotrophs in *E. coli*. The responsible genes have since been sequenced and sufficient genetic (Martin & Mulks, 1992; Sakanyan *et al.*, 1992) and enzymic (Sakanyan *et al.*, 1993a) data have been accumulated to prove that these organisms possess a bifunctional *argJ*-encoded product capable of using both the acetyl group of *N*-acetylornithine and that of acetylCoA to acetylate glutamate. It appears that *Bacillus subtilis* also harbours a bifunctional acetyltransferase (O'Reilly & Devine, 1994).

Although the *argJ* gene by itself would thus be able to assure both the first and the fifth steps of arginine biosynthesis in these organisms, there is genetic evidence for the existence of an independent functional *argA* gene in *N. gonorrhoeae* (Picard & Dillon, 1989; Martin & Mulks, 1992) and enzyme data for *B. stearothermophilus* point in the same direction (Sakanyan *et al.*, 1992). In

Pseudomonas aeruginosa, however, the synthase and transacetylase activities can be separated by gel filtration (Haas *et al.*, 1972). Moreover, *argA* mutants have been isolated which display a normal acetyltransferase but no synthase activity and hence an arginineless phenotype. It has been shown that the synthase enzymes of *P. aeruginosa* (Haas *et al.*, 1972; Haas & Leisinger, 1974) and *Saccharomyces cerevisiae* (Wipf & Leisinger, 1979) lack ornithine acetyltransferase activity; the properties of the ornithine acetyltransferases were not studied in detail. The data for *P. aeruginosa* therefore suggest the existence of a monofunctional ornithine acetyltransferase in this organism. Similarly, the cloned *Streptomyces coelicolor* ornithine acetyltransferase gene complements *E. coli argE* but not *argA* mutants (Hindle *et al.*, 1994).

In some micro-organisms the metabolic flow through the acetyl cycle is controlled by arginine-mediated feedback inhibition of the second biosynthetic step, catalysed by *N*-acetylglutamate kinase (ATP: *N*-acetyl-L-glutamate 5-phosphotransferase; EC 2.7.2.8; Udaka, 1966; Hoare & Hoare, 1966; Haas & Leisinger, 1975; Meile & Leisinger,

Table 1. Bacterial strains and plasmids used in this study

Strain/plasmid	Relevant genotype/phenotype	Reference/source
<i>E. coli</i> K12		
JM109	e14 ⁻ (McrA ⁻) <i>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 Δ(lac-proAB)</i> [F' <i>traD36 proAB lacI^qΔM15</i>]	Yanisch-Perron <i>et al.</i> (1985)
XA4	F ⁻ <i>argA nalA λ⁻ λ^s hsdR</i>	Mountain <i>et al.</i> (1984)
XB25	F ⁻ <i>argB nalA λ⁻ λ^s hsdR</i>	Mountain <i>et al.</i> (1984)
XC33	F ⁻ <i>argC nalA rpoB λ⁺ hsdR</i>	Mountain <i>et al.</i> (1984)
PT2	F ⁻ <i>argD proAB his ilvA metB rpsL</i>	Mountain <i>et al.</i> (1984)
XF	F ⁻ Δ101 (<i>proAB-argF-lac</i>) <i>argI thi supE λ⁻ λ^s hsdR hsdM</i>	Mountain <i>et al.</i> (1984)
XG31	Hfr <i>argG his rpoB rpsL λ⁻ λ^s hsdR</i>	Mountain <i>et al.</i> (1984)
X190	Hfr(P4X) <i>carA metB thr λ⁺ zjj202::Tn10 hsdR</i>	Mountain <i>et al.</i> (1984)
XJEF8	Hfr(P4X) <i>carB metB thr λ⁺ zjj202::Tn10 hsdR</i>	Mountain <i>et al.</i> (1984)
XS1D2R	F ⁻ Δ(<i>ppc-argE</i>) <i>nalA rpoB λ⁻ hsdR recA</i>	Sakanyan <i>et al.</i> (1992)
XA4argE	As XA4, but <i>argE86::Tn10</i>	Sakanyan <i>et al.</i> (1992)
<i>C. glutamicum</i> ATCC 13032	Wild-type	Collection of Industrial Microorganisms, Moscow, Russia
Plasmids		
pBR327	Ap ^r Tc ^r	Bolivar (1978)
pUC9	Ap ^r <i>lacZ</i>	Vieira & Messing (1984)
pUC18	Ap ^r <i>lacZ</i>	Yanisch-Perron <i>et al.</i> (1985)
pGA46	Ap ^r Cm ^r	An & Friesen (1979)
pBluescript II KS(+)	Ap ^r <i>lacZ</i>	Stratagene
pPP2	pBR327-derivative, Ap ^r Tc ^r	This work

1984). However, in *B. stearothermophilus* no noticeable inhibition of *N*-acetylglutamate kinase by either ornithine or arginine could be detected. Instead, the target for inhibition was found to be the bifunctional *argJ* (and possibly the *argA*) gene product: both *N*-acetylglutamate synthase and ornithine acetyltransferase activities were strongly inhibited by ornithine. Arginine, however, did not affect either activity (Sakanyan *et al.*, 1993a). Consequently, in this organism the metabolic intermediate ornithine, rather than the end-product arginine appears to be critical for controlling metabolite conversions in the arginine acetyl cycle.

In order to clarify the organization and the regulation of the cyclic acetylation pathway a more extensive study was desirable. We have undertaken the genetic and enzymic examination of the pathway in *Corynebacterium glutamicum*, formerly *Micrococcus glutamicus* (Jones & Collins, 1986), a Gram-positive mesophilic bacterium. Indigenous non-pathogenic corynebacteria, particularly representatives of *Corynebacterium* and *Brevibacterium*, synthesize and excrete large quantities of glutamic acid in broth cultures (Shio *et al.*, 1962). Genetically improved strains have therefore long been exploited for the industrial production of arginine and proline (Kinoshita & Nahayama, 1978), but the underlying genetics has not been studied extensively. The data reported below provide new information on the acetyl cycle in this organism.

METHODS

Bacterial strains and plasmids. The strains and plasmids used in this work are listed in Table 1.

Media and growth conditions. *C. glutamicum* cells were grown on a rotary shaker (150 r.p.m.) at 30 °C in Luria-Bertani (LB) medium (Sambrook *et al.*, 1989) or in a synthetic medium described by Bröer *et al.* (1993). LB as well as the synthetic M9 medium (Miller, 1972) were used for *E. coli* K12 strains. M9 was supplemented with all auxotrophic requirements other than arginine for complementation analysis of *arg* mutants by *C. glutamicum* DNA. *E. coli* cells were grown at 37 °C, except for complementation tests which were performed at 30 °C. Antibiotics were added at a final concentration of 50 µg ml⁻¹ for ampicillin, 30 µg ml⁻¹ for chloramphenicol and 25 µg ml⁻¹ for tetracycline.

DNA manipulation and transformation. Chromosomal DNA of *C. glutamicum* was isolated as described by Eikmanns *et al.* (1991). Plasmid DNA from *E. coli* was isolated by the alkaline lysis method of Birnboim & Doly (1979) or with Qiagen-tip columns (QIAGEN). Agarose gel electrophoresis, DNA restriction, alkaline phosphatase treatment and ligation were performed following classical protocols (Sambrook *et al.*, 1989). *E. coli* strains were transformed following the CaCl₂ procedure (Sambrook *et al.*, 1989) or by electroporation with the geneZapper 450-2500 apparatus (International Biotechnologies) according to the manufacturer's recommendations.

DNA sequencing and sequence analysis. Prior to sequencing subfragments were cloned into the pBluescript II KS(+) vector.

ExoIII-generated unidirectional deletions were obtained according to the method of Sambrook *et al.* (1989). Sequencing was performed by the dideoxynucleotide chain-termination method (Sanger *et al.*, 1977) using sequenase Quick-denaturate plasmid sequencing kits (USB) and [α - 35 S]dATP (Amersham). Either universal or reverse primers or synthetic 17-mer oligonucleotides (provided by D. Gigot, Research Institute, CERIA-COOVI, Brussels, and by Eurogentec) were used. Sequence ladders were resolved on gels containing 6% (w/v) polyacrylamide with taurine. The sequence data were compiled, analysed and aligned with various programs from the MacVector package (International Biotechnologies), as well as with CLUSTAL V (Higgins & Sharp, 1988) and FASTA (Pearson & Lipman, 1988). The Swiss-Prot database (EMBL) was consulted for protein sequences.

Enzyme assays. *E. coli* and *C. glutamicum* cells were grown in synthetic media, harvested by centrifugation during the exponential phase, washed in 0.9% (w/v) NaCl and resuspended in 10 mM potassium phosphate buffer (pH 6.5) containing 15% (v/v) glycerol, 1 mM EDTA, 1 mM DTT and 2 mM PMSF for *N*-acetylglutamate synthase and ornithine acetyltransferase assays, or in 25 mM Tris/HCl buffer (pH 7.5) for other assays. Cells were disrupted by sonication and the resulting crude extract was centrifuged (20000 *g*, 15 min). All these treatments were performed at temperatures below 10 °C. Enzyme assays were carried out at 30 °C. For inhibition experiments the crude cell extracts were passed through Sephadex G-25 columns equilibrated with extraction buffer.

N-Acetylglutamate synthase and ornithine acetyltransferase were assayed as described in Van de Casteele *et al.* (1990), except that 15% (v/v) glycerol, 10 mM MgCl₂, and 7 mM aminoxyacetic acid were added to the incubation mixture. *N*-Acetylglutamate kinase was measured by the ferric chloride method (Udaka, 1966) as described previously by Van de Casteele *et al.* (1990). Acetylornithinase was measured by the method of Vogel & McLellan (1970) as described in Sakanyan *et al.* (1993c).

RESULTS AND DISCUSSION

Cloning of *C. glutamicum* arginine biosynthetic genes and their expression in *E. coli* mutants

Cloning of the *C. glutamicum argJ* gene was undertaken by selecting for heterologous complementation of *argE* deficiency in *E. coli* as obtained with *N. gonorrhoeae* and *B. stearothermophilus* DNA (Picard & Dillon, 1989; Sakanyan *et al.*, 1990). An *EcoRI*-digest of *C. glutamicum* ATCC 13032 DNA was ligated into *EcoRI*-cleaved vector pBR327. The resulting plasmids were transformed into *E. coli* K12 XS1D2R and arginine prototrophs were selected on synthetic medium supplemented with succinate, ampicillin and tetracycline. The recombinant pPPP2 plasmid which carries a single 4.4 kb *EcoRI* insert was isolated from the selected transformants. Its restriction map derived from single- and double digest data with several enzymes is shown in Fig. 2.

Ampicillin-resistant pPPP2 transformants of various arginine auxotrophic *E. coli* K12 mutants were screened for complementation by replica plating on synthetic medium without arginine. Apart from the *argE*-deficient strain mentioned above, pPPP2 complemented *argB* mutant XB25. Of particular interest to our study is its failure to complement *argA*-deficient strains XA4 and its derivative

XA4*argE*. Enzyme assays summarized in Table 2 confirmed these results. *N*-Acetylglutamate kinase and ornithine acetyltransferase activities measured in cell extracts of *E. coli* K12 XB25(pPP2) and *E. coli* XA4(pPPP2), respectively, were raised significantly above the background level of the plasmidless strain. Acetylglutamate synthase and acetylornithinase activities, in contrast, remained undetectable in extracts of *E. coli* K12 XA4(pPPP2) and *E. coli* K12 XS1D2R(pPPP2). From these results it can be inferred that the cloned fragment harbours the structural *C. glutamicum argB* and *argJ* genes and that the *argJ*-encoded ornithine acetyltransferase relieves the acetylornithinase deficiency of the *E. coli argE* mutant by heterologous complementation.

Since the native *C. glutamicum* ATCC 13032 displays *N*-acetylglutamate synthase activity (see Table 2) it can be assumed that the corresponding *argA* gene is not located on the pPP2 plasmid unless it is non-functional in *E. coli* cells. Consequently, the cloned *C. glutamicum argJ* gene appears to encode a monofunctional enzyme capable of transacetylating the acetyl group of *N*-acetylornithine (ornithine acetyltransferase activity), but not that of acetylCoA (*N*-acetylglutamate synthase activity) to glutamate.

Sequence analysis

The sequence of the entire 4.4 kb insert of *C. glutamicum* DNA in pPPP2 has been established on both strands using *ExoIII*-generated 200–300 bp deletions in a subcloned 1.9 kb *HindIII*–*XhoI* fragment (plasmid pKS-1.9) and several other subcloned fragments. Analysis of the nucleotide sequence reveals five large ORFs (Fig. 3) oriented in the same way, those at the fragments' ends being truncated. The ORFs were numbered as indicated in Fig. 2. Comparison of the corresponding amino acid sequences with known arginine metabolic enzymes from various sources (see also below) shows that all of them correspond to arginine biosynthetic genes: truncated ORF1 matches the C-terminal end of the *argC*-encoded polypeptide, ORF 2, ORF3 and ORF4 appear respectively homologous with the *argJ*-, *B*- and *D*-encoded enzymes and finally, the truncated ORF5 corresponds with the N-terminal region of the *argF* gene product. Putative RBSs can be found upstream of the proposed initiation codons of ORFs 2–5 and are indicated in Fig. 3. The cloned 4.4 kb insert therefore seems to contain an important part of a large arginine biosynthetic cluster as in *B. subtilis* (Mountain *et al.*, 1986), *N. gonorrhoeae* (Picard & Dillon, 1989), *B. stearothermophilus* (Sakanyan *et al.*, 1990; Sakanyan *et al.*, 1993a) and *S. coelicolor* (Hindle *et al.*, 1994). A fairly large ORF6 oriented in the opposite direction showed no significant resemblance to any sequence as yet registered in the protein databases. Searching specifically for homology between *C. glutamicum* ORFs and the known acetylglutamate synthase sequences of *E. coli* (Brown *et al.*, 1987), *P. aeruginosa* and *Pseudomonas putida* (Dharmsthiti & Krishnapillai, 1993) and *Neurospora crassa* (Y. Yu & R. L. Weiss, unpublished; EMBL accession number L35484) was negative, except for ORF3 which

Fig. 2. Genetic and restriction maps of the 4.4 kb insert of *C. glutamicum* DNA in pPP2. The arrowhead boxes show the localization and orientation of the ORFs. The corresponding genes and the encoded enzymes are indicated. Plasmid pEX4 was constructed by cloning the *EcoRI*-*XhoI* fragment from pPP2 into *EcoRI*/*SalI*-digested pUC9, and plasmid pGAB4 was constructed by cloning the *HindIII*-*XhoI* fragment into *HindIII*/*SalI*-digested pGA46. These plasmids were used in complementation experiments with *E. coli* K12 XB25 and XS1D2R strains (results indicated to the right). The *HindIII*-*XhoI* fragment from pPP2 was also cloned into the *HindIII*/*SalI* sites of pUC18 and re-cloned after excision with *HindIII* and *EcoRI* (taking advantage of the polylinker site) into *HindIII*/*EcoRI*-digested pBluescript II KS(+), yielding plasmid pKS-1.9.

corresponds to the *argB* gene. This homology between acetylglutamate synthase and acetylglutamate kinase is supported by the comparison of other *argA* and *argB* gene couples (Reith & Munholland, 1993; see below).

According to a BESTFIT comparison, the *C. glutamicum* ArgJ sequence shares 36, 35 and 39% identical amino acids with the ornithine acetyltransferases of *N. gonorrhoeae* (Martin & Mulks, 1992), *B. subtilis* (O'Reilly & Devine, 1994) and *B. stearothermophilus* (Sakanyan *et al.*, 1993a), respectively. The CLUSTAL alignment shown in Fig. 4 indicates that the similarity covers the whole sequence, though the *C. glutamicum* polypeptide appears shorter than its bifunctional homologues by 11–12 amino acids at the N-terminal end. Its predicted molecular mass is 39.8 kDa, approximately 3 kDa less than for the other known bacterial ornithine acetyltransferases; it contains only 6.7% of basic amino acids which is substantially lower than the 10% found for the other ArgJ products.

A BESTFIT comparison shows that the *N*-acetylglutamate kinase polypeptide sequence of *C. glutamicum* shares approximately 42% identical amino acids with the *N*-acetylglutamate kinase of *Porphyra umbilicalis* (Reith & Munholland, 1993), 39% with that of *B. stearothermophilus* (Sakanyan *et al.*, 1993b), 35% with that of *B. subtilis* (O'Reilly & Devine 1994), 29% with that of *E. coli* (Parsot *et al.*, 1988), 25% with that of *N. crassa* (Gessert *et al.*, 1994) and 23% with those of *S. cerevisiae* (Boonchird *et al.*, 1991) and *Schizosaccharomyces pombe* (Van Huffel *et al.*, 1992).

Sequence analysis indicates that the initiation codon of the *C. glutamicum argD* gene is contiguous to the *argB* TAA termination codon, whereas an intergenic space is

observed between the other corynebacterial *arg* genes of the cluster. The same tendency was observed in *B. stearothermophilus* (Sakanyan *et al.*, 1993b) and *B. subtilis* (O'Reilly & Devine, 1994), where the *argB* and *argD* genes overlap for a few nucleotides and suggests translational coupling, while a relatively long intergenic space was found at the *argC/argJ* and *argJ/argB* transitions. A CLUSTAL alignment of the deduced ArgD polypeptide sequence with those of *E. coli*, *B. subtilis* and *S. cerevisiae* (data not shown) revealed 40, 42 and 36% identical amino acids, respectively.

The overall G+C content of the 4.4 kb stretch of *C. glutamicum* DNA amounts to 54.6%, which corresponds with the mean value for glutamic-acid-producing corynebacteria (Yamada & Komagata, 1970). A remarkable feature in codon usage is the unexpectedly high appearance of the rare CGA arginine codon in the *argJ* gene (4 out of 12 codons). Apart from that there is a relatively low occurrence of G+C in the third position of the *argJ* codons: 51.7% as compared to 59.3 and 64.1% for the *argB* and *argD* genes, respectively. There is no strong bias for corynebacterial 'preferred' codons (Malumbres *et al.*, 1993) in either *argJ*, *argB* or *argD*; therefore a low-to-moderate expression might be expected.

Evidence for a secondary promoter upstream of the *argB* gene

From the sequence analysis it appears that the pEX4 plasmid, constructed by subcloning the 3.1 kb *EcoRI*-*XhoI* fragment in *EcoRI*/*SalI*-cut pUC9 vector, should carry the whole of the structural information for both the

argC
 I L T T A T A P L K E G V T A E Q A R
 G A A T T C T C A C C A C T G C A A C C G A C T T T G A A A G A A G C G T T A C C G C A G A A C G G C T C G C G 60
 A V Y E E F Y A Q E T F V H V L P E G A
 C A G T A T A T A A G A G T T C T A T G C A C A G A A C C T T C G T G C A T G T C T T C C A G A A G T G C A C 120
 Q P Q T Q A V L G S N M C H V Q V E I D
 A G C C A A A C C A A G C A G T T C T G G C T C C A C A T G T G C C A G T G A G A A T T G A T G 180
 E E A G K V L V T S A I D N L T K G T A
 A G G A A G C A G C A A G T C T T G T T A C T C C G C A A T G A T A C C T C A C A A G G A A C T C G C G 240
 G A A V Q C M N L S V G F D E A A G L P
 G G C C C G T G T T C A G T G C A T G A A C T T A A G C G T T G G T T T G A T G A G G C A G C A G C C T G C C A C 300
 Q V G V A P *
 A G G T C G G C G T C G A C C T T A A G T A G C G C T T A A A G C G G C T T C A A A C C A A G C C C T A A 360
argI
 M A E K G I
 C C A G A A A C A C A A A C A C A T C A T T A T C A G T A C C A G T T C C A C T G C A G A A A A G G C A T 420
 T A P K G F V A S A T T A G I K A S G N
 T A C G C G C C G A A A G G C T T G T T G C T C A C A C A G C C G G G T A T T A A G C T T C G G C A A 480
 P D M A L V T V N Q G P E F S A A A V F T
 T C C T G A C A T G G C G T T G T G T T A A C A G G T C A G A G T T T C C G A G C G C G C G T G T T A C 540
 R N R V F A A P V K V S R E N V A D G Q
 A C G T A C C C A G T T T T C G A C G C C T G T A A G G T G A G C G A G A A C G T T G C T G A T G C C A 600
 I R A V L Y N A G N A N A C N G L Q G E
 G A T C A G G C G T T T T G T A C A C C T G G T A A T G C T A T G C T G T A A T G T T C C A G G T G A 660
 K D A R E S V S H L A Q N L G L E D S D
 G A A G G A T C G C T G A G T C G T T T C A T C A T G C T A A A A T T G G C T T G A G A G A T T C C G A 720
 I G V C S T G L I G E L L P M D K L N A
 T A T G T G T G T G T G T C A C T G G C T A T T A T G T G A G T G C T C C G A T G A T A A G C T C A A T G C 780
 G I D Q L T A E A P L G D N G A A A A K
 A G G T A T T G A T C A G T G A C C C G T G A G C A C C T T T G G T G A C A A T G G T C A G C T G C T G C A A 840
 A I M T T D T V D K E T V V F A D G W T
 G C G T A C T G A C C A C T G A C C G T G A T A A G G A A A C C G T C G T G T T G C T G A T G T G A C 900
 V G G M G K G V G M M A P S L A T M L V
 T G T C G G C G A A T G G C A A G G C G T G G C A T G A T G G C C G C T C T T G C C A C C A T G C T G T 960
 C L T T D A S V T Q E M A Q I A L A N A
 C T G C T T G A C C A C T G A T C C G T T A C T G A G A A T G G C T C A G A T C C G C T G C T G A T G C 1020
 T A V T F D T L D I D G S T S T N D T V
 T A C G C C G T T A C G T T G A C A C C T G G A T T G A T G A T C A A C T C C A C C A A T G A C A C C G T 1080
 F L L A S G A S I T P T Q D E L N D A
 G T T C T G C T G G C A C T G G C G T A G C G A A T C A C C C A C T C A G A T G A A C T C A A C A G T G C 1140
 V Y A A C S D I A A K L Q A D A E G V T
 G G T G A C C A C G C T G T T T C T G A T A T C G A G C G A A G C T T C A G C T G A T G C A G A G S T G T G A C 1200
 K R V A V T V V G T T N N E Q A I N A A
 C A A G C G C G T T G C T G A C A G T G G T G G A A C C A A C A A C A G A C G A G C A T T A A T G C G C G 1260
 R T V A R D N L F K C A M F G S D P N W
 T C G C A C T G T G C T G T G A C A A T T T G T C A A G T C G C A A T G T T G G A T C T G A T C C A A A C T G 1320
 G R V L A A V G M A D A D M E P E K I S
 G G G T C G C G T T G T T C T G A T A T C G A G C G A A G C T T C A G C T G A T G C A G A G S T G T G A C 1380
 V F F N G Q A V C L D S T G A P G A R E
 T G T G T C T T A A T G T C A A G C A T G T G C T T G A T T C C A C T G C G C T C C T G T G C T G T G A 1440
 V D L S G A D I D V R I D L G T S G E G
 G G T G A T C T T T C G G C G T G A C A T T A T G T C G A A T G A T T T G G C C A C C A T G G G A A G G 1500
 Q A T V R T T D L S F S Y V E I N S A Y
 C A G G C A A C A G T T G A A C C A C T G A C T G A G C T T C T C A C T G G A G A T C A A C T C C G C G T A 1560
 S S *
 C A G C T C T T A A A A A A A C A G C A C T C C A A C T A A C A A G C A G G A A A A G C G C A C A G G C A T G A A 1620
 D L I K D L G S E V R A N V L A E A L P
 T G A C T T G A T C A A A G A T T A G G C T T G A G G T G C G C A A A T G C T C G C T G A G C G T T G C C 1680
 W L Q H F R D K I V V V K Y G G N A M V
 A T G G T G C A G C A C T T C C C G C A A A G A T T G T G T G T G A A A T A T G C G G A A C G C C A T G G T 1740
 D D D L K A A F A A D M V F L R T V G A
 G G A T G A T C A A A G C T G C T T T T G T G C G A C A T G G T C T T T G C G A C C G T G G G A G G 1800
 K P V V V H G G G P Q I S E M L N R V G
 A A A A C C A G T G G T G C A C G G T G T G A C T C A G A T T T C T G A G A T C T A A A C G T G T G G G 1860
 L Q G E F K G G F R V T T P E V M D I V
 T C T C C A G G C G A G T T C A A G G T G G T T T C C G T G T G A C C A C C T C T G A G G T C A T G G A C A T T G T 1920
 R M V L F G Q V G R D L V G L I N S H G
 G C G C A T G T C T T T G G T C A G T C G G C G C A T T A G T T G G T T T G A T C A A C T C T A T G G 1980
 P Y A V A G L T A Q K R M
 C C C T T A C G T G T G G A A C C T C G G T G A G A T G C G G C C T G T T T A C C G C A G A A G C G C A T 2040
 V N I D G V P T D I G L V G D I I N V D
 G G T C A A C A T C G A T G G C G A C C A C C A T T G G T T T G G T G G A G A C A T C A T T A A T G T C G A 2100
 A S S L M D I I E A G R I P V V S T I A
 T G C C T C T C T T G A T G A T A T C A T C G A G C C G T G C A T T C C T G T G T C T A C G A T T G C 2160
 P G E D G Q I Y N I N A D T A A G A L A
 T C C A G C G A G A C G C C A G A T T T A C A A C A T T A A C G C C A T A C C G A C A G G T G C T T G G C 2220
 A A I G A E R L L V L T N V E G L Y T D
 T G C A G G A T T G G T G C A A C C C T G T G G T T C C A C A A T G T G A A G G T C T G T A C C G A 2280
 W P D K S S L V S K I K A T E L E A I L
 T T G C C T G A T A A G A G C T C A C T G G T G T C A A G A T C A A G C C A C C G A C T G G A G C C A T T C T 2340
 P G L D S G M I P K M E S C L N A V R G
 T C C G G A C T T G A T C C G C A T G A T T C A A A G A T G G A G T G T G C T T G A A C G C G T G C G T G G 2400
 G V S A A H V I D G R I A H S V L L E L
 G G G A G T A A G C C T G C T C A T G T C A T T G A C G C C G A T C G C G A C T C G G T G T G C T G G A G C T 2460
 L T M G G I G T M V L P D V F D R E N Y
 T T T G A C C A T G G G T G A A T T G G C A C A T G G T G C C G A T G T T T T G A T C G G G A G A A T T A 2520
 P E G T V F R K D D K D G E L * M S T L
 T C C T G A A G G C A C C G T T T T A G A A A A G A C A A G G A T T G G G A C T G T A A T G A G C A C G C T 2580
 E T W P Q V I I N T Y G T P P V E L V S
 G G A A C T T G G C C A C A G G T C A T T A T A A C T G C G C A C C C A C C A G T T G A G C T G G T G T C 2640
 G K G A T V T D D Q G N V Y I D L L A G
 C G C A A G G C G A A C C G T C A T G A T G A C C A G G C A A T G T C A T C G A T C T G C T G C G G G 2700
 I A V N A L G H A H P A I E A V T N Q
 C A T C G A G T C A A C G G T T G G C C A C C C A C C G G C A T C A T C G A G C G G T C A C C A C C A 2760
 I G Q L G H V S N L F A S R P V V E V A
 G A T C G G C C A C T T G G T C A C G T C T C A A C T T G T T G C A T C A G C C C G T C G T C A G G T G C C 2820
 E E L I K R F S L D D A T L A A Q T R V
 C A G A G A G C T C A T C A A G C G T T T T C G C T T G A C G A C C C C C T C G C G C G A A A C C G G G T 2880
 F F C N S G A E A N E A A F K I A R L T
 T T T C T T C G C A C T C G G G C G C A A G C A A C A G A G G T G C T T T C A A G A T T C T G A G T T G A C 2940
 G R S R I L A A V H G F H G R T M G S L
 T G G T C G T C C C G A T T C T G G T C A G T C A T G G T T T C C A C G C C C A C C A T G G T T C C C T 3000
 A L T G Q P D K R E A F L P M P S G V E
 C G C G T A C T G G C A G C A G A A C C G T G A A C G T T C C T G C A A T G C C A A G C G G T G G A 3060
 F Y P Y G D T D Y L R K M V E T N P T D
 G T T C T A C C C T T A C G C G C A C C A G G A T T A C T T G C G A A A A T G A A A C C A A C C A A C G G A 3120
 V A A I F L E P I Q G E T G V V P A P E
 T G T G G C T G C A T C T C T G A G C C A A T C C A G G G T A A A C G G G C G T T G T T C C A G C A C T G A 3180
 G F L K A V R E L C D E Y G I L M I T D
 A G G A T T C C T C A A G G C A G T G C G C A G C T G T G C A T G A G T A G A C C A T C T T G A T G A C C G A 3240
 E V Q T G V G R T G D F F A H Q H D G V
 T G A A G T C C A G A C T G G C G T T G C G G T A C C G G C A T T T C T T T G C A C A T C A G C A G T G C G T 3300
 V P D V V T M A K G L G G G T I G A C
 T G T C C G A T G T G G T A C C A T G C C A A G G A C T T G G C G C G C T T C C C A T C G G T C T G 3360
 L A L R A A E L M T P G K H G T T F G G
 T T T G C A C T G C G T C A G C T G A A T T A T G A C C C A G G A A G C A G C C A C C A C T T T C G T G G 3420
 N P V A C A A A K A V L S V V D D A F C
 C A A C C A G T T G C T T G T G C A G C T G C C A A G G C A G T G C T G T C T G T G T G A T A C G C T T T C T G 3480
 A E V A R K Q L F K E L L A K V D G V V
 C G C A A A G T T C C C G C A A G C A G C T G T T C A A G G A C T T T T G C C A A G G T T G A C G G C T T G T 3540
 D V R G R G L M L G V V L E R D V A K Q
 A G A C T C C G T G G C A G G G C T T G A T G T T G G G C T G G T G G A G C G C G A C T G C A A A G C A 3600
 A V L D G F K H G V I L N A P A D N I I
 A G C T G T T T G A T G G T T T T A A G C A C G C G T T A T T T G A A T G C A C C G C G A A A C A T T 3660
 R L T P P L V I T D E E I A D A V K A I
 C C G T T T G A C C C C G C G T G T A C C A C C A G A A A T C C G A C C A G C C A G T C A A G C T A T 3720
 A E T I A *
 T G C G A G A C A A T C G C A A A A G C A C T C A A C T A T G A C T T C A A C C A C A G G T T G C C A T T 3780
 F L A D D D L T P A E Q A E V L T L A A
 T T C T G C C T G A T A T G A T C A C C C T G C A G C A G C A G A G G T T T G A C C C T A G C C G A A 3840
 K L K A A P F S E R P L E G P K S V A V
 A G C T C A A G G C A G C C G C T T T C G A G C G T C C A C T G A G G A C C A A A G T C C G T C A G T T C 3900
 L F D K T S T R T R F S F D A G I A H L
 T T T T G A T A A G A C T C A A C T C G A T C G C T T C C T T C G A C G C G G C A C T C G C T A T T T G G 3960
 G G H A I V V D S G S S O M G K G E S L
 G T G G A C A C C A T C G T G T G A T T C C G T A G C T C A C A G A T G G T A A G G C G A G T C C C T G C 4020
 Q D T A A V L S R Y V E A I V W R T Y A
 A G G A C A C C G A C T G A T A T G T C C C G T A C G T G G A A G C A A T T G T G T G C C A C C A T A C G C A C 4080
 H S N F H A M A E T S T V P L V N S L S
 A C A G C A A T T C C A C G C C A T G C G G A G A C G T C C A C T G T G C C G C T G G T G A A C T C C T T G T C C G 4140
 D D L H P C Q I L A D L Q T I V E N L S
 A T G A T T G C A C C C A T G C C A G A T T C T G G C T G A T C T G C A G A C T A T C T G G A A A C C T C A G C 4200
 P E E G P A G L K G K K A V I D G D
 C T G A A A G G C C A G A G C C T T A A G G T A A A A G C T G T G T A C T G G C G A T G C G A C A 4260
 N N M A N S Y M I G F A T A G M D I S I
 A C A A C A T G C C A A C C T C A T A T G A T T G G C T T T G C A C C C G G G C A T G G A T A T T C C A T C A 4320
 I A P E G F O P R A E F
 T C G C T C C T G A A G G T T C C A G C C T G C T G C G A A T T C 4355
EcoRI

Fig. 3. For legend see facing page.

C.gl.	MA-----EKGITAPKGFVASATTAGIKASGNPDMAVNVQGP	38
B.st.	MTITKQTGQVTAADGTVTTPGEGFAAGVNAGLRY-S-KNDLGVILCDVPA	49
B.sub.	M-IQLSEDIIVKVT-GDVSSPKGPAQGVHCGRLYS-KKDLGVIISETPA	47
N.g.	MAVNLTEKTAELPDIDIGALYTAQAGVKKPG-----HTDLTLTAVAAGS	45
C.gl.	FSAAAVFTRNRVFAAPVKVSRENVDGQ-IRAVLYNAGNANACNGLQGEK	87
B.st.	-SAAAVYTQSHFQAAPLKVTQASLAVEQKLQAVIVNRPCANACTGAQGLK	98
B.sub.	-VSAAVYTQSHFQAAPIKVTQDSLKHGPTLKAVIVNSAIAANACTGEGGLK	96
N.g.	-TVGAFTTNRFCAPPAVIAKSHLFDEGVRALVINTGNANACTGAQGR	94
C.gl.	DARESVSHLAQNLGLESDIGVCTGLIGELLPMKDLNAGIDQLTAEAPL	137
B.st.	DAYEMRELCAKQFGLALHHVAVASTGVIGEYLPMEKIRAGIKQLVPGVTM	148
B.sub.	DAYTMRESFASQIGIEPELVAVSSTGVIGELHMEKIHAGIELLKE--TP	144
N.g.	DALAVCAAAARQIGCKPNVNFSTGVILEPLPADKIIAALPKMPAPFWN	144
C.gl.	GDNGAAAKAAMITTDVTDKET---VVFADGWTV--GGMGKGVGMPPSLA	182
B.st.	ADAEAFQ-TAILTTDTVMKRCYQTTIDGK-TVTVGGAAGSGMIHPNMA	196
B.sub.	AGSGDFE-EAILTTDTVIKQTCVELAIGGK-TVTIGGARKSGSMIHPNMA	192
N.g.	EAA----RAIMTTDTVPKAASREGKVGQHTVTRATVIAKSGSMIHPNMA	189
C.gl.	TMLVCLTTDASVTQEMAIQALANATAVTFDTLDIDGSTSTNDTVFLLASG	232
B.st.	TMLAFITTDANVSSPVLHAALRSITDVSFNQITVDGDTSTNDMVMVMSG	246
B.sub.	TMLGFVTTDAAIEEKALQKALRETTDVSFNQITVDGDTSTNDMVLVMA	242
N.g.	TMLGFATDAKVSQPVQLQMTQEIADETFTNTITVDGDTSTNDSFVIIATG	239
C.gl.	ASGIT-----PTQDELNDVYAACSIAAKLQADAEVGTKRVAVTVVG	275
B.st.	LAGNDELTP-DHPDWFNYEALRKTCEDLAKQIAKDGEATKLEIVRVRG	295
B.sub.	CAENELTE-DHPDWPVFKKALLTCEDELAKEIARDGEGATKLEIAQVQG	291
N.g.	KNSQSEIDMIADPRYAQLKELLSLALELAQAIVRDGEATKFTITVRVEN	289
C.gl.	TTNNEQAINAARTVARDNLFKCAMFGSDPNWGRVLAAGVMAD-ADMEPEK	324
B.st.	AKTDEEAKKIAKQIVGSNLVKTAVYGADANWGRIIIGAIGYSD-AEVPNDN	344
B.sub.	AKNNLDANVIAKKIVGSNLVKTAVYGTANWGRIIIGAIGHSA-AQVTAAE	340
N.g.	AKTCDEARQAAYAAARSPLVKTAFASDPNLGKRLAAIGYADVADLDTDL	339
C.gl.	ISVFFNGQAVCLDSTGAPAREVD---LSGADIDVRIDLGTSGEGQATV	370
B.st.	VDVAIGPMVMLK-GSEPPPFSEEEAAYLQGETVIVIEVDL-HIGDGVGVA	392
B.sub.	VEVYLGGQCLFK-NNEPPPFSEISIAKEYLEGDEITIVIKM-ABGDGNRA	388
N.g.	VEVYLLDDILVAEHGGRASYSYEAQGVMSKDEITVRIKL-HRQQAATV	388
C.gl.	RTTDLFSFSVVEINSAYSS	388
B.st.	WGCDLTVDYVRIKINASYRT	410
B.sub.	WGCDLTVDYIKINASYRT	406
N.g.	YTCDLSHGVSINADYRS	406

Fig. 4. Comparison of entire amino acid sequences of ornithine acetyltransferases by CLUSTAL V alignment. C.gl., *C. glutamicum* (this work); B.st., *B. stearothermophilus* (Sakanyan *et al.*, 1993a); B.sub., *B. subtilis* (O'Reilly & Devine, 1994); N.g., *N. gonorrhoeae* (Martin & Mulks, 1992). Asterisks indicate that identical residues occur in all four polypeptides; dots show replacement by similar amino acids.

argJ and the *argB* genes (Fig. 2). Nevertheless, no *E. coli* K12 XS1D2R(pEX4) transformants could be selected on arginineless synthetic medium supplemented with ampicillin. Arginine prototrophic *E. coli* K12 XB25(pEX4) transformants, however, were readily obtained.

The orientation of the *C. glutamicum argJ* gene, in opposition to the *lac* promoter-directed transcription in pEX4, is probably responsible for the contrasting *argE* complementation results obtained with pPP2 and pEX4. The ornithine acetyltransferase activity displayed by pPP2 harbouring *E. coli* strains would then result from some promoter located in the pBR327 vector itself.

Transcription of the *C. glutamicum argB* gene in *E. coli*, however, seems independent from an extraneous promoter, a result confirmed by the significant *N*-acetylglutamate kinase activity observed in the *E. coli* K12 XB25(pGAB4) strain (Table 2). The pGAB4 plasmid was constructed by inserting the 1.9 kb *HindIII*-*XhoI* fragment (see Fig. 2) in *HindIII*/*SalI* double-digested promoter-probe vector pGA46. As no transcription can proceed from the pGA46 vector into the inserted DNA (An & Frisen, 1979), it seems likely that in *E. coli* cells at least, transcription can be initiated at a promoter located between the *HindIII* site at 1172 nt and the beginning of the structural *argB* gene of *C. glutamicum*. Evidence for the occurrence of a promoter site recognized by *E. coli* RNA polymerase preceding the *argB* structural gene has been reported for *B. stearothermophilus* as well (Sakanyan *et al.*, 1993b).

Repression of enzyme formation and feedback inhibition by arginine

N-Acetylglutamate synthase, *N*-acetylglutamate kinase and ornithine acetyltransferase activities were measured in extracts of *C. glutamicum* cells grown in the absence or in the presence of arginine (Table 2). The levels of *N*-acetylglutamate synthase and ornithine acetyltransferase were not affected by arginine addition. A fivefold repression of *N*-acetylglutamate kinase synthesis was observed in the presence of arginine.

Inhibition of enzyme activity by arginine was tested for the three enzymes by adding L-arginine (concentration range 0.01–100 mM) to the reaction mixture of the enzyme assays described above. Arginine was found to inhibit *N*-acetylglutamate synthase and *N*-acetylglutamate kinase activities; the arginine concentrations for 50% inhibition were 40 and 2 mM, respectively. Feedback inhibition by arginine of corynebacterial *N*-acetylglutamate kinase has already been reported (Udaka, 1966). Inhibition by L-arginine (more than 80% at 10 mM arginine) could also be detected for *C. glutamicum N*-acetylglutamate kinase synthesized in *E. coli* K12 *argB* mutant cells carrying pPP2, pKS-1.9 or pGAB4. Whether *N*-acetylglutamate kinase inhibition actually plays a regulatory role *in vivo* remains to be established, since the apparent inhibition constant of arginine is high. One must, however, take into account that this value was determined with crude extracts and at saturating levels of substrates.

Inhibition by ornithine was tested for the enzymes listed in Table 2, except acetylornithinase. No inhibition could be detected for the first or the second biosynthetic enzymes, either in *C. glutamicum* or in recombinant *E. coli* cell extracts. The corynebacterial ornithine acetyltransferase activity was inhibited by ornithine (product inhibition); the apparent K_i value at saturating levels of substrates was 5 mM.

Fig. 3. Nucleotide sequence of the 4.4 kb DNA region of *C. glutamicum* ATCC 13032 with the predicted amino acid sequences encoded by the *argCJBDF* genes cluster. The potential ribosome-binding site (RBS) and selected restriction sites referred to in Fig. 2 are underlined. Stop codons are marked with asterisks.

Table 2. Specific activities of four arginine biosynthetic enzymes in *C. glutamicum* and in *E. coli* strains carrying *C. glutamicum* *arg* genes

Values are the means of at least two measurements made on independent cultures. The values for replicate assays differed from the mean by < 20 % for *N*-acetylglutamate synthase and ornithine acetyltransferase, and by < 10 % for the other activities.

Strain	Arginine addition*	Specific activity [units (mg protein) ⁻¹]			
		<i>N</i> -Acetylglutamate synthase	<i>N</i> -Acetylglutamate kinase	Ornithine acetyltransferase	Acetyl-ornithinase
<i>C. glutamicum</i> ATCC 13032	—	0.06	0.10	0.16	ND
	+	0.06	0.02	0.12	ND
<i>E. coli</i> K12					
XA4	+	< 0.001	ND	< 0.001	ND
XA4(pPP2)	+	< 0.001	ND	0.36	ND
XB25	+	ND	< 0.005	ND	ND
XB25(pPP2)	—	ND	2.1	ND	ND
XB25(pPP2)	+	ND	2.1	ND	ND
XB25(pKS-1.9)	—	ND	2.8	ND	ND
XB25(pKS-1.9)	+	ND	1.3	ND	ND
XB25(pGAB4)	—	ND	2.5	ND	ND
XB25[pBluescript II KS(+)]	+	ND	< 0.005	ND	ND
XS1D2R	+	ND	ND	ND	< 0.001
XS1D2R(pPP2)	—	ND	ND	ND	< 0.001

ND, Not determined.

* Arginine was added to synthetic medium at a concentration of 5 mM. Succinate (0.5 %, w/v) was added for *E. coli* XS1D2R strains. *E. coli* strains carrying plasmids were grown in the presence of ampicillin.

Evolutionary relationships between enzymes of the acetyl cycle

Comparison based on the Pearson & Lipman algorithm (1988) fails to reveal any significant similarity between the sequences of *C. glutamicum* ornithine acetyltransferase and known *N*-acetylglutamate synthases. This result enforces our earlier suspicion that in spite of their functional relatedness the *argA* and *argJ* gene products belong to different evolutionary families (Sakanyan *et al.*, 1993a).

FASTA comparisons (Pearson & Lipman, 1988) with the registered polypeptide sequences do not point to any clear affiliation of the ornithine acetyltransferases. On the other hand similarities between the N-terminal part of ArgA and the ArgB polypeptide sequence have recently been noticed (Reith & Munholland, 1993; Gessert *et al.*, 1994). These similarities appear in fact most pronounced when *E. coli* ArgA and *C. glutamicum* ArgB amino acid sequences are compared: application of the Pearson & Lipman (1988) RDF2 program establishes a similarity value that is 15 standard deviations above the mean value obtained with 100 random permutations of either sequence, a highly significant value. It may therefore be assumed that acetylglutamate synthases and kinases are indeed evolutionarily related.

An alignment of the known ArgA and ArgB polypeptide sequences (data not shown) indeed reveals several highly conserved amino acids. Surprisingly, no direct relatedness

could be detected between the acetylglutamate synthase of *N. crassa* (Y. Yu & R. L. Weiss; EMBL accession number L35484) and those of other organisms: the *N. crassa* ArgA enzyme seems weakly similar to kinase sequences only. The other known acetylglutamate synthase polypeptide sequences are obviously longer than the kinases by a stretch of about 100–140 amino acid residues, and some highly conserved regions occur in this part. The results of a FASTA search of the registered sequences in the Swiss-Prot database suggests an intriguing affiliation. Indeed, a high similarity between this region of the *E. coli* acetylglutamate synthase and a 153 amino acid ORF, found next to the *trpGDC* cluster in *Azospirillum* (Zimmer *et al.*, 1991), which in its turn can be related to the *E. coli* RimI enzyme that acetylates the N-terminus of the ribosomal S18 gene (Yoshikawa *et al.*, 1987) and the *Streptomyces lavendulae* StaT protein, an acetylCoA-dependent acetyltransferase (Horinouchi *et al.*, 1987). The *Azospirillum* ORF is, moreover, highly similar to the *E. coli* PhnO protein (Makimo *et al.*, 1991), which is involved in alkylphosphonate utilization that again appears related to various other acetyltransferases. These similitudes might reflect an ancient link between the *argA*-encoded enzyme C-terminal part and a family of small acetylCoA-dependent acetyltransferases.

New insights into the function and the evolution of the acetyl cycle are presently being looked for in a comparative analysis of structure–function relationships

in the monofunctional ornithine acetyltransferases of *C. glutamicum* disclosed in this work and the bifunctional, homologous enzyme of *B. stearotheophilus* (Sakanyan *et al.*, 1993a).

ACKNOWLEDGEMENTS

We are indebted to D. Gigot for the synthesis of oligodeoxyribonucleotides. This work was partially supported by a Grant from the Région des Pays de la Loire (Contrat de Plan Etat-Région), by the Belgian Foundation for Fundamental and Joint Research (FRFC-FKFO), and by an OZR-fund of the Vrije Universiteit Brussel.

REFERENCES

- An, G. & Friesen, J. D. (1979). Plasmid vehicles for direct cloning of *Escherichia coli* promoters. *J Bacteriol* **140**, 400–407.
- Birnboim, H. C. & Doly, J. (1979). A rapid alkaline extraction procedure for screening recombinant DNA. *Nucleic Acids Res* **7**, 1513–1523.
- Bolivar, F. (1978). Construction and characterization of new cloning vehicles. III. Derivatives of plasmid pBR322 carrying unique *EcoRI* sites for selection of *EcoRI* generated recombinant DNA molecules. *Gene* **4**, 121–130.
- Boonchird, C., Messengey, F. & Dubois, S. (1991). Characterization of the yeast ARG5,6 gene: determination of the nucleotide sequence, analysis of the control region and of ARG5,6 transcript. *Mol & Gen Genet* **226**, 154–166.
- Bröer, S., Eggeling, L. & Krämer, R. (1993). Strains of *Corynebacterium glutamicum* with different lysine productivities may have different lysine excretion systems. *Appl Environ Microbiol* **59**, 316–321.
- Brown, K., Finch, P. W., Hickson, I. D. & Emmerson, P. T. (1987). Complete nucleotide sequence of the *Escherichia coli* *argA* gene. *Nucleic Acids Res* **15**, 10586.
- Cunin, R., Glansdorff, N., Pierard, A. & Stalon, V. (1986). Biosynthesis and metabolism of arginine in bacteria. *Microbiol Rev* **50**, 314–352.
- Davis, R. H. (1986). Compartmental and regulatory mechanisms in the arginine pathways of *Neurospora crassa* and *Saccharomyces cerevisiae*. *Microbiol Rev* **50**, 280–313.
- Dharmsthiti, S. & Krishnapillai, V. (1993). DNA sequence conservation at the gene level in a conserved chromosomal segment in two *Pseudomonas* species. *J Genet* **72**, 1–14.
- Eikmanns, B. J., Kircher, M., Liebl, W. & Reinscheid, D. J. (1991). Discrimination of *Corynebacterium glutamicum*, *Brevibacterium flavum* and *Brevibacterium lactofermentum* by restriction pattern analysis of DNA adjacent to the *hom* gene. *FEMS Microbiol Lett* **82**, 203–208.
- Gessert, S. F., Kim, J. H., Nargang, F. E. & Weiss, R. L. (1994). A polypeptide precursor of two mitochondrial enzymes in *Neurospora crassa*. Gene structure and precursor processing. *J Biol Chem* **269**, 8189–8203.
- Haas, D. & Leisinger, T. (1974). Multiple control of N-acetylglutamate synthetase from *Pseudomonas aeruginosa*: synergistic inhibition by acetylglutamate and polyamines. *Biochem Biophys Res Commun* **60**, 42–47.
- Haas, D. & Leisinger, T. (1975). N-acetylglutamate 5-phosphotransferase of *Pseudomonas aeruginosa*. Catalytic and regulatory properties. *Eur J Biochem* **52**, 377–383.
- Haas, D., Kurer, V. & Leisinger, T. (1972). N-acetylglutamate synthetase of *Pseudomonas aeruginosa*. An assay *in vitro* and feedback inhibition by arginine. *Eur J Biochem* **31**, 290–295.
- Higgins, D. G. & Sharp, P. M. (1988). CLUSTAL: a package for performing multiple sequence alignment on a microcomputer. *Gene* **73**, 237–244.
- Hindle, Z., Callis, R., Dowden, S., Rudd, B. A. M. & Baumberg, S. (1994). Cloning and expression in *Escherichia coli* of a *Streptomyces coelicolor* A3(2) *argCJB* gene cluster. *Microbiology* **140**, 311–320.
- Hoare, D. S. & Hoare, S. L. (1966). Feedback regulation of arginine biosynthesis in blue-green algae and photosynthetic bacteria. *J Bacteriol* **92**, 375–379.
- Horinouchi, S., Furuya, K., Nishiyama, M., Suzuki, H. & Beppu, T. (1987). Nucleotide sequence of the streptothricin acetyltransferase gene from *Streptomyces lavendulae* and its expression in heterologous hosts. *J Bacteriol* **169**, 1929–1937.
- Jones, D. & Collins, M. D. (1986). Irregular, nonsporing Gram-positive rods. In *Bergey's Manual of Systematic Bacteriology*, vol. 2, pp. 1261–1434. Edited by P. H. A. Sneath, N. S. Mair, M. E. Sharpe & J. G. Holt. Baltimore: Williams & Wilkins.
- Kinoshita, S. & Nakayama, K. (1978). Amino acids. In *Primary Products of Metabolism*, pp. 209–261. Edited by A. H. Rose. London, New York & San Francisco: Academic Press.
- Makino, K., Kim, S., Shinagawa, H., Amemura, M. & Nataka, A. (1991). Molecular analysis of the cryptic and functional *phn* operons for phosphate use in *Escherichia coli* K-12. *J Bacteriol* **173**, 2665–2672.
- Malumbres, M., Gil, J. A. & Martin, J. F. (1993). Codon preference in *Corynebacteria*. *Gene* **134**, 15–24.
- Martin, P. R. & Mulks, M. H. (1992). Sequence analysis and complementation studies of the *argJ* gene encoding ornithine acetyltransferase from *Neisseria gonorrhoeae*. *J Bacteriol* **174**, 2694–2701.
- Meile, L. & Leisinger, T. (1984). Enzymes of arginine biosynthesis in methanogenic bacteria. *Experientia* **40**, 899–900.
- Miller, J. H. (1972). *Experiments in Molecular Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Mountain, A., Mann, N. H., Munton, R. N. & Baumberg, S. (1984). Cloning of a *Bacillus subtilis* restriction fragment complementing auxotrophic mutants of eight *Escherichia coli* genes of arginine biosynthesis. *Mol & Gen Genet* **197**, 82–89.
- Mountain, A., McChesney, J., Smith, M. C. M. & Baumberg, S. (1986). Gene sequence encoding early enzymes of arginine synthesis within a cluster in *Bacillus subtilis*, as revealed by cloning in *Escherichia coli*. *J Bacteriol* **165**, 1026–1028.
- O'Reilly, M. & Devine, K. M. (1994). Sequence and analysis of the citrulline biosynthetic operon *argC–F* from *Bacillus subtilis*. *Microbiology* **140**, 1023–1025.
- Parsot, C., Boyen, A., Cohen, G. N. & Glansdorff, N. (1988). Nucleotide sequence of *Escherichia coli* *argB* and *argC* genes: comparison of N-acetylglutamate kinase and N-acetylglutamate- γ -semialdehyde dehydrogenase with homologous and analogous enzymes. *Gene* **68**, 275–283.
- Pearson, W. R. & Lipman, D. J. (1988). Improved tools for biological sequence comparison. *Proc Natl Acad Sci USA* **85**, 2444–2448.
- Picard, F. J. & Dillon, J. R. (1989). Cloning and organization of seven arginine biosynthesis genes from *Neisseria gonorrhoeae*. *J Bacteriol* **171**, 1644–1651.
- Reith, M. & Munholland, J. (1993). Two amino-acid biosynthetic genes are encoded on the plasmid genome of the red alga *Porphyra umbilicalis*. *Curr Genet* **23**, 59–65.

- Sakanyan, V. A., Hovsepyan, A. S., Mett, I. L., Kochikyan, A. V. & Petrosyan, P. K. (1990). Molecular cloning and structural-functional analysis of the arginine biosynthesis genes of the thermophilic bacterium *Bacillus stearothermophilus*. *Genetika* **26**, 1915–1925.
- Sakanyan, V., Kochikyan, A., Mett, I., Legrain, C., Charlier, D., Piérard, A. & Glansdorff, N. (1992). A re-examination of the pathway for ornithine biosynthesis in a thermophilic and two mesophilic *Bacillus* species. *J Gen Microbiol* **138**, 125–130.
- Sakanyan, V., Charlier, D., Legrain, C., Kochikyan, A., Mett, I., Piérard, A. & Glansdorff, N. (1993a). Primary structure, partial purification and regulation of key enzymes of the acetyl cycle of arginine biosynthesis in *Bacillus stearothermophilus*: dual function of ornithine acetyltransferase. *J Gen Microbiol* **139**, 393–402.
- Sakanyan, V. A., Legrain, C., Charlier, D., Kochikyan, A. V., Osina, N. K. & Glansdorff, N. (1993b). N-acetylglutamate-5-phosphotransferase of the thermophilic bacterium *Bacillus stearothermophilus*: nucleotide sequence and enzyme characterization. *Genetika* **29**, 556–570.
- Sakanyan, V., Desmarez, L., Legrain, C., Charlier, D., Mett, I., Kochikyan, A., Savchenko, A., Boyen, A., Falmagne, P., Piérard, A. & Glansdorff, N. (1993c). Gene cloning, sequence analysis, purification, and characterization of a thermostable aminoacylase from *Bacillus stearothermophilus*. *Appl Environ Microbiol* **59**, 3878–3888.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Sanger, F., Nicklen, S. & Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA* **74**, 5463–5467.
- Shiio, I., Otsuka, S. I. & Takahashi, M. (1962). Effect of biotin on the bacterial formation of glutamic acid. I. Glutamate formation and cellular permeability of amino acids. *J Biochem* **51**, 56–62.
- Shinners, E. N. & Catlin, B. W. (1978). Arginine biosynthesis in *Neisseria gonorrhoeae*: enzymes catalyzing the formation of ornithine and citrulline. *J Bacteriol* **136**, 131–135.
- Udaka, S. (1966). Pathway-specific pattern of control of arginine biosynthesis in bacteria. *J Bacteriol* **91**, 617–621.
- Udaka, S. & Kinoshita S. (1958). Studies on L-ornithine fermentation. I. The biosynthetic pathway of L-ornithine in *Micrococcus glutamicus*. *J Gen Appl Microbiol* **4**, 272–282.
- Van de Castele, M., Demarez, M., Legrain, C., Glansdorff, N. & Piérard, A. (1990). Pathways of arginine biosynthesis in extreme thermophilic archaeo- and eubacteria. *J Gen Microbiol* **136**, 1177–1183.
- Van Huffel, C., Dubois, E. & Messenguy, F. (1992). Cloning and sequencing of *arg3* and *arg11* genes of *Schizosaccharomyces pombe* on a 10-kb DNA fragment. Heterologous expression and mitochondrial targeting of their translation products. *Eur J Biochem* **205**, 33–43.
- Vieira, J. & Messing, J. (1982). The pUC plasmids, a M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. *Gene* **19**, 259–268.
- Vogel, H. J. & MacLellan, W. L. (1970). Acetylornithinase (*E. coli*). *Methods Enzymol* **17A**, 265–269.
- Wipf, B. & Leisinger, T. (1979). Regulation of activity and synthesis of N-acetylglutamate synthase from *Saccharomyces cerevisiae*. *J Bacteriol* **140**, 874–880.
- Yamada, K. & Komagata K. (1970). Taxonomic studies on coryneform bacteria. III. DNA base composition of coryneform bacteria. *J Gen Appl Microbiol* **16**, 55–65.
- Yanisch-Perron, C., Vieira, J. & Messing, J. (1985). Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* **33**, 103–119.
- Yoshikawa A., Isono, S., Sheback, A. & Isono, K. (1987). Cloning and nucleotide sequencing of the genes *rimI* and *rimJ* which encode enzymes acetylating ribosomal proteins S18 and S5 of *Escherichia coli*. *Mol & Gen Genet* **209**, 481–488.
- Zimmer, W., Aparicio, C. & Elmerich, C. (1991). Relationship between tryptophan biosynthesis and indole-3-acetic acid production in *Azospirillum*: identification and sequencing of a *trpGDC* cluster. *Mol & Gen Genet* **229**, 41–51.

Received 1 August 1995; accepted 25 August 1995.