Genes and enzymes of the acetyl cycle of arginine biosynthesis in the extreme thermophilic bacterium *Thermus thermophilus* HB27

Margot Baetens,^{1,†} Christianne Legrain,³ Anne Boyen^{1,2} and Nicolas Glansdorff^{1,2,3}

Author for correspondence: Nicolas Glansdorff. Tel: +32 2 526 72 75. Fax: +32 2 526 72 73.
e-mail: ceriair@ulb.ac.be

Microbiologie, Vrije Universiteit Brussel¹, Vlaams Interuniversitair Instituut voor Biotechnologie² and Research Institute CERIA-COOV³, Emile Grysonlaan 1, B-1070 Brussel, Belgium

An arginine biosynthetic gene cluster, *argC-argJ*, of the extreme thermophilic bacterium *Thermus thermophilus* HB27 was isolated by heterologous complementation of an *Escherichia coli* acetylornithinase mutant. The recombinant plasmid (pTHM1) conferred ornithine acetyltransferase activity to the *E. coli* host, implying that *T. thermophilus* uses the energetically more economic pathway for the deacetylation of acetylornithine. pTHM1 was, however, unable to complement an *E. coli argA* mutant and no acetylglutamate synthase activity could be detected in *E. coli argA* cells containing pTHM1. The *T. thermophilus argJ*-encoded enzyme is thus monofunctional and is unable to use acetyl-CoA to acetylate glutamate (contrary to the *Bacillus stearothermophilus* homologue). Alignment of several ornithine acetyltransferase amino acid sequences showed no obvious pattern that could account for this difference; however, the monofunctional enzymes proved to have shorter N-termini. Sequence analysis of the pTHM1 3.2 kb insert revealed the presence of the *argC* gene (encoding *N*-acetylglutamate-5-semialdehyde dehydrogenase) upstream of the *argJ* gene. Alignment of several *N*-acetylglutamate-5-semialdehyde dehydrogenase amino acid sequences allowed identification of two strongly conserved putative motifs for cofactor binding: a putative FAD-binding site and a motif reminiscent of the NADPH-binding fingerprint. The relationship between the amino acid content of both enzymes and thermostability is discussed and an effect of the GC content bias is indicated. Transcription of both the *argC* and *argJ* genes appeared to be vector-dependent. The *argJ*-encoded enzyme activity was twofold repressed by arginine in the native host and was inhibited by ornithine. Both upstream of the *argC* gene and downstream of the *argJ* gene an ORF with unknown function was found, indicating that the organization of the arginine biosynthetic genes in *T. thermophilus* is new.

Keywords: *Thermus thermophilus*, *argCJ* sequence, ornithine acetyltransferase, *N*-acetylglutamate-5-semialdehyde dehydrogenase, regulation

INTRODUCTION

Thermus thermophilus is an extreme thermophilic bacterium growing optimally at 72 °C and up to 85 °C.

One of the first organisms of this type to have been isolated (Oshima & Imahori, 1974), it has been used ever since as a model system for studies on thermophily. As part of a general programme focused on the

Abbreviations: OAT, ornithine acetyltransferase; NAGSD, *N*-acetylglutamate-5-semialdehyde dehydrogenase.

† Present address: Department of Microbiology and Immunology, Sherman Fairchild Science Building, 299 Campus Drive, Stanford University, 94305-5124, USA.

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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.

molecular physiology of thermophilic prokaryotes, we have embarked upon the enzymic and genetic characterization of the arginine and pyrimidine systems in *Thermus* (Van de Castele, 1994; Van de Castele *et al.*, 1990, 1994, 1997a, b).

Among primary metabolic systems arginine biosynthesis is notable for its complexity and variability at the genetic level, and by its connection with several other pathways such as pyrimidine and polyamine biosynthesis, and diverse degradative pathways (see Cunin *et al.*, 1986; Glansdorff, 1996). Moreover, the pattern of arginine synthesis is not unique (see below), a rare instance in biosynthetic metabolism.

The initial steps of the arginine biosynthetic pathway proceed via *N*-acetylated intermediates (Fig. 1). Two alternative ways have evolved to split off the acetyl group from *N*-acetylornithine: in the linear pathway, which is found in the *Enterobacteriaceae* (Cunin *et al.*, 1986) and in *Sulfolobus solfataricus* (Van de Castele *et al.*, 1990) acetylornithinase (EC 3.5.1.16, encoded by *argE*) catalyses the hydrolysis of *N*-acetylornithine into the arginine precursor ornithine and acetate. All other prokaryotes and the eukaryotic microbes investigated up to now use an energetically more economic pathway which recycles the acetyl group onto glutamate, yielding acetylglutamate. This reaction is catalysed by ornithine acetyltransferase (OAT, EC 2.3.1.35, encoded by *argJ*).

In these organisms the first step in the pathway fulfils an anaplerotic role: once *N*-acetylornithine is synthesized the action of *N*-acetylglutamate synthase (encoded by *argA*) becomes superfluous.

In *Bacillus stearothermophilus* (Sakanyan *et al.*, 1993) the *argJ* gene product is bifunctional: the enzyme is able to use both acetyl-CoA and *N*-acetylornithine to acetylate glutamate, thus expressing both *N*-acetylglutamate synthase and OAT activities. Also, in *Neisseria gonorrhoeae* (Martin & Mulks, 1992) and in *Bacillus subtilis* (O'Reilly & Devine, 1994) the *argJ* gene was shown to complement both *argA* and *argE* *Escherichia coli* auxotrophs. In *Saccharomyces cerevisiae*, the ARG7 gene (the yeast *argJ* homologue) is able to complement both yeast ARG2 and ARG7 mutants as well as *E. coli argA* and *argE* mutants; the corresponding protein displays acetylglutamate synthase activity when overexpressed (Crabeel *et al.*, 1997).

On the other hand, in *Pseudomonas aeruginosa*, the two enzyme activities are separable by gel filtration (Haas & Holloway, 1977); the *argJ* gene product of *Streptomyces coelicolor* is unable to complement an *E. coli argA* mutant (Hindle *et al.*, 1994) and in *Corynebacterium glutamicum* the *argJ* gene was shown to encode a monofunctional enzyme displaying only OAT activity (Sakanyan *et al.*, 1996).

In the micro-organisms utilizing the linear pathway, the first enzyme in the pathway – *N*-acetylglutamate synthase – is the target for feedback inhibition by arginine (Vyas & Maas, 1963). Since in the organisms which recycle the acetyl group the first enzyme fulfils an anaplerotic role, it is the second enzyme of the pathway (*N*-acetylglutamate-5-phosphotransferase) which is usually the subject of feedback inhibition by arginine (see Cunin *et al.*, 1986; Davis, 1986). However, in *Thermus* ZO5 only OAT was feedback-inhibited by the pathway's end product (Van de Casteele *et al.*, 1990). In *B. stearothermophilus*, *N*-acetylglutamate-5-phosphotransferase is not feedback-inhibited either. Instead, it is also the OAT activity which is inhibited, although not by arginine but by ornithine (Sakanyan *et al.*, 1993). This was also observed for *C. glutamicum* OAT (Sakanyan *et al.*, 1996) and for its *Sacch. cerevisiae* homologue (Crabeel *et al.*, 1997). *Sulf. solfataricus* (using the linear pathway) and *Thermotoga maritima* (using the cyclic pathway) appear devoid of feedback inhibition (Van de Casteele *et al.*, 1990) under the conditions applied.

The organization of the arginine biosynthetic genes differs greatly amongst the different systematic groups. In *P. aeruginosa* and *N. gonorrhoeae* the *arg* genes are not clustered at all (Haas & Holloway, 1977; Picard & Dillon, 1989). In the *Enterobacteriaceae*, an *argECBH* cluster is found in *E. coli* (Glansdorff, 1965) (where the *argE* and *argCBH* genes are transcribed divergently from converging promoters facing each other over a common control region; Elseviers *et al.*, 1972; Pouwels *et al.*, 1974); an *argECBH* cluster is also found in

Salmonella typhimurium (Sanderson, 1970) and an *argECBGH* cluster is present in *Proteus mirabilis* and *Serratia marcescens* (Prozesky, 1968; Matsumoto *et al.*, 1975). The other *arg* genes are scattered over the chromosome. In *B. subtilis* two operons are found: *argCJBD-cpa-argF* and *argGH* (Mountain *et al.*, 1986; Harwood & Baumberg, 1977); in *B. stearothermophilus* there is an *argCJBD* operon (Sakanyan *et al.*, 1993; Savchenko *et al.*, 1996), in *C. glutamicum* an *argCJBDF* cluster (Sakanyan *et al.*, 1996) and in *Strep. coelicolor* an *argCJB* one (Hindle *et al.*, 1994). In these last three organisms nothing is known about possible further downstream arginine genes. The data of Ludovice *et al.* (1992) suggest that in *Streptomyces clavuligerus* *argC* and *argJ* are linked. In *Vibrio* 2693 there exists an *argCBFGH* gene organization (Liang *et al.*, 1996) and there are indications that upstream of this cluster a divergently expressed *argE*, and downstream an *argA* gene are present (Liang, 1997; Z. Liang, Y. Xu & N. Glansdorff, unpublished).

From the above it appears that further studies on arginine metabolism in *T. thermophilus* are justified from several convergent points of view. The organism is not only a thermophilic paradigm in the Bacterial domain but it also belongs to a deeply branching line of descent on the 16S RNA life tree (Woese, 1987; Williams, 1992). This suggests that, in general, comparisons of gene structure and gene organization between *Thermus* and other prokaryotes could be particularly interesting. In the case of arginine biosynthesis, which has also gained the status of a model system in molecular physiology, our present knowledge remains limited to the identification of the pathway (Van de Casteele *et al.*, 1990); no genetic data are available. Regarding *argJ* in particular, it is not known whether this gene encodes a mono- or a bifunctional enzyme. In this paper we have characterized the gene encoding the acetylornithine deacetylating enzyme of *T. thermophilus*; it is the first gene of this kind to have been described in an extreme thermophilic bacterium. We have also examined some of the regulatory properties of the cognate enzyme and carried out its partial purification. The *argC* gene encoding *N*-acetylglutamate-5-semialdehyde dehydrogenase (NAGSD, EC 1.2.1.38) was found to be adjacent to *argJ*.

METHODS

Bacterial strains and plasmids. *T. thermophilus* HB27 wild-type strain is described by Oshima & Imahori (1974) and was obtained from Dr Y. Koyama. *hsd E. coli* arginine auxotrophs XA4 (*argA*), XC33 (*argC*) and XS1D2 (*argE*) are described by Mountain *et al.* (1984) and were kindly provided by Dr S. Baumberg. XA4*argE* is described by Sakanyan *et al.* (1992) and was constructed in this laboratory. The *E. coli* plasmid vector pUC19 (Messing & Vieira, 1982) was purchased from Boehringer Mannheim and pBluescript II KS(+) (Short *et al.*, 1988) from Stratagene.

Culture conditions. *E. coli* strains were grown at 37 °C in L broth and in synthetic 132 medium (Falmagne *et al.*, 1965). Succinate was added to 0.25% (w/v) where necessary.

Concentrations of antibiotics were 25 µg ampicillin ml⁻¹ and 20 µg tetracycline hydrochloride ml⁻¹. *T. thermophilus* HB27 was grown at 65 °C in defined 162 arginine- and uracil-free (AUF) medium (Piérard & Wiame, 1964; Degryse *et al.*, 1978) with pyruvate (20 mM) and ammonium sulphate (10 mM) as carbon and nitrogen sources, respectively.

DNA manipulation and transformation. Small-scale plasmid preparations were performed by the cleared lysate method according to Birnboim & Doly (1979). Large-scale preparations were recovered with Nucleobond AX PC100 (Macherey-Nagel). Restriction enzymes and T4 ligase were purchased from Boehringer Mannheim and used according to the manufacturer's recommendations. *E. coli* cells were made competent for transformation by CaCl₂ treatment (Sambrook *et al.*, 1989).

DNA sequencing strategy. The entire 3.2 kb *Hind*III fragment of plasmid pTHM1 was cloned in pBluescript KS(+) in two orientations. Overlapping deletions were generated by use of exonuclease III and S1 nuclease as described by Sambrook *et al.* (1989). Preparation of single-stranded DNA template was according to the method of Messing (1983), using R408 as a helper phage. The sequences of both strands were established by the dideoxy chain-termination method (Sanger, 1977). To solve ambiguities in sequence reading due to band compression we substituted the analogue C⁷dGTP for dGTP in the sequencing mixes (Pharmacia).

Enzyme assays. OAT and *N*-acetylglutamate synthase were assayed as described by Van de Castele *et al.* (1990) at 65 °C. One enzyme unit is defined as the amount of enzyme that converts 1 µmol substrate to product per hour. Protein concentrations were determined by the Lowry method.

Computer analysis. Database searches and sequence analyses were performed using the BLAST program (Altschul *et al.*, 1990), the CLUSTAL W program (Thompson *et al.*, 1994) and the GCG package from the University of Wisconsin (Devereux *et al.*, 1984).

Partial purification of OAT. Frozen cells of *T. thermophilus* from a 15 litre culture (13.5 g wet mass) were thawed in 35 ml potassium phosphate buffer (pH 7.5; hereafter referred to as phosphate buffer) supplemented with DNase (5 µg ml⁻¹) and disrupted by sonication for 15 min in a Raytheon sonic oscillator (250 W, 10 kHz). Intact cells and debris were removed by two consecutive centrifugations (20 000 *g*, 30 min). The supernatant was heated to 95 °C for 15 min and cleared of the precipitate by centrifugation (20 000 *g*, 20 min). Solid ammonium sulphate was added to the supernatant to 45% saturation, the solution was stirred for 1 h and centrifuged at 20 000 *g* for 30 min. The pellet was suspended in phosphate buffer and extensively dialysed. The dialysed solution (12.5 ml) was loaded on a DEAE-Sepharose XK 16/20 column (Pharmacia), which was washed with 150 ml phosphate buffer and eluted with a linear gradient (400 ml total volume, 50–500 mM potassium phosphate buffer, pH 7.5). Active fractions were pooled and concentrated (to 1.2 ml) by ultrafiltration through an Amicon YM-10 membrane. This concentrated material was applied to a gel filtration Sephadex G-200 SF column (XK 26/40, Pharmacia) and eluted with phosphate buffer. Fractions (1.4 ml) were collected at 5 ml h⁻¹. The gel filtration column was calibrated using the following standards: catalase (*M_r* 232 000), aldolase (*M_r* 158 000), albumin (*M_r* 67 000), ovalbumin (*M_r* 43 000), chymotrypsinogen A (*M_r* 25 000) and ribonuclease A (*M_r* 13 700).

RESULTS

Cloning of *T. thermophilus* arginine biosynthetic genes and their expression in *E. coli*

We cloned the *T. thermophilus* gene responsible for the deacetylation of acetylornithine by heterologous complementation of an *E. coli* *argE* mutant (XS1D2), as was done for *B. stearothermophilus* (Sakanyan *et al.*, 1990), *N. gonorrhoeae* (Martin & Mulks, 1992) and *C. glutamicum* (Sakanyan *et al.*, 1996). *Hind*III-digested *T. thermophilus* chromosomal DNA was ligated in a *Hind*III-digested and dephosphorylated pUC19 vector. The resulting plasmids were transformed into *E. coli* K-12 XS1D2 and selection for arginine prototrophs was performed on synthetic medium supplemented with succinate and ampicillin. A recombinant plasmid, pTHM1, complementing the *E. coli* XS1D2 auxotrophy was found to contain a 3.2 kb insert. This plasmid was tested for its ability to complement other *E. coli* arginine mutants. An *argC* mutant (XC33) could be complemented, but pTHM1 was unable to restore prototrophy in an *argA* mutant (XA4) or an *argAargE* double mutant (XA4argE).

Enzymic assays showed that pTHM1 conferred OAT activity to the *E. coli* XS1D2 mutant [5.6 units (mg protein)⁻¹, as compared to 1.3 units (mg protein)⁻¹ in *T. thermophilus* extract], thus indicating that *T. thermophilus* uses the cyclic pathway for deacetylation of *N*-acetylornithine. No *N*-acetylglutamate synthase activity could be detected in the *E. coli* XA4 host harbouring pTHM1, corroborating the complementation results. The *argJ* gene product of *T. thermophilus* thus appears to be monofunctional, as in *Pseudomonas*, *Streptomyces* and *Corynebacterium* (see Introduction).

Sequence analysis

Analysis of the nucleotide sequence of the pTHM1 3.2 kb insert (Fig. 2) revealed the presence of four ORFs, of which the first and last ones were truncated. Comparison of the corresponding amino acid sequences with known arginine metabolic enzymes from different sources allowed us to identify the second ORF as encoding NAGSD (*argC*) and the third one as encoding OAT (*argJ*) (Table 1). For the corresponding amino acid sequences of the truncated ORFs 1 (468 nt, 156 aa) and 4 (520 nt, 173 aa), no homology with any arginine biosynthetic gene and no significant homology with any protein in the SWISSPROT database (BLASTX and BLASTP programs) could be found.

The second ORF, encoding *argC*, is 1032 nt long. Putative start codons are two GTGs at position 465 and 474, respectively. In organisms having a high G+C content GUG codons are very commonly found (e.g. *Micrococcus luteus*, 70–75.5 mol% G+C, Ohama *et al.*, 1987; *Streptomyces* sp., approximately 73 mol% G+C, Zalacain *et al.*, 1986). Only 2 nt 5' to the most proximal GTG codon (and 11 nt from the second one) we found a potential ribosome-binding site (5' GGAGGTG) complementary to the 3' end sequence of

Fig. 2. Organization of the 3.2 kb *Hind*III fragment of pTHM1 carrying the *argC* and *argJ* genes of *T. thermophilus*. The proposed ribosome-binding sites are indicated by dots and the putative promoter by a bent arrow.

Table 1. Percentage amino acid identity and similarity of *T. thermophilus argC* and *argJ* gene products and the corresponding gene products in several other organisms (BESTFIT program)

NR, Not relevant; U, sequence unavailable.

	ArgC		ArgJ	
	% similarity	% identity	% similarity	% identity
<i>E. coli</i>	60.6	38.7	NR	NR
<i>N. gonorrhoeae</i>	U	U	60.5	41.8
<i>Synechocystis</i> sp.	64.6	42.6	64.3	47.3
<i>Anabaena</i> sp.	54.4	32.0	U	U
<i>B. subtilis</i>	1.1	42.2	62.8	43.0
<i>B. stearothermophilus</i>	62.9	45.5	64.5	42.3
<i>C. glutamicum</i>	U	U	57.9	38.1
<i>Strep. clavuligerus</i>	63.7	43.2	U	U
<i>M. jannaschii</i>	66.4	42.7	64.2	44.6
<i>Sacch. cerevisiae</i>	57.3	32.0	57.1	38.9

T. thermophilus 16S rRNA (5' GAUCACCUCCU-UUCU, Hartmann & Erdmann, 1989). In *E. coli* and *B. subtilis* a 2 nt spacing would result in negligible expression of *lacZ* (Vellanoweth & Rabinowitz, 1992) and an 11 nt spacing would reduce expression severely. However, in *Thermus* a few examples are known of spacings that appear to be as short as 2 or as long as 14 nt (database accession numbers M33159 and D17669). The CLUSTAL W multiple sequence alignment of nine NAGSD enzymes (Fig. 3) favours the GTG at position 474 as possible start codon. Unequivocal elucidation of the start codon will be obtained after purification of the corresponding protein and N-terminal amino acid sequencing.

ORF3, comprising the predicted *argJ* gene, is 1143 nt long. Nucleotides 1499–1501 encode the ATG start codon; 4 nt upstream a putative Shine–Dalgarno sequence is present (5' AAGGAGG). The stop codon of *argC* overlaps with the translational start of *argJ*. The organization of these genes would allow translational coupling. A similar arrangement was observed with *T. thermophilus* HB27 *trpB* and *trpA* genes (Koyama & Furukawa, 1990) and *T. thermophilus* HB8 *pheS* and *pheT* genes (Kreutzer *et al.*, 1992), and occurs at many other prokaryotic operons whose gene products are required in equimolar amounts. In *C. glutamicum* (Sakanyan *et al.*, 1996), *B. stearothermophilus*

(Sakanyan *et al.*, 1993) and *B. subtilis* (O'Reilly & Devine, 1994) an intergenic space between the *argC* and *argJ* genes is present.

In ORF4 an ATG codon is present at position 2714. Four nucleotides upstream a potential RBS is found (5' AGGTGA). Thirty-three nucleotides further upstream a putative Pribnow box is found (5' TATCCT), preceded by a putative –35 sequence at a distance of 17 bp (5' TTGACA). These sequence data thus suggest the presence of a promoter in front of ORF4.

The stop codon of the *argJ* gene overlaps with the putative –35 sequence upstream of ORF4. Downstream of the *argJ* gene stop codon no obvious termination signal is present.

Upstream of the *argC* gene no obvious promoter sequence can be distinguished. When the 3.2 kb fragment was cloned in the opposite orientation in pUC19, the resulting plasmid was no longer capable of complementing an *E. coli argE* mutant; moreover, S1 nuclease mapping of a fragment comprising the N-terminus of the *argC* gene and an upstream region failed to indicate a transcription start point (data not shown). This indicates that in *E. coli* the *argJ* and *argC* genes on pTHM1 are not expressed from a promoter located on the *T. thermophilus* fragment but from a vector-encoded one.

T.thermo.	-----VGILGASGYGAELRLRLKAHPE
E.coli	-----MLNTLIVGASGYGAELVTYVNRHPH
Synecho.	-----MGNEKARVGIIGASGYGGIQLVRLLEHPQ
Anabaena	-----MKNPKIFIDEGAGTGLQIYSRLNERDD
B.subt.	-----MKIGIVGATGYGGTELVRILSHPH
B.stearo.	-----MMNVAIGATGYSGAELFRLLYGHPH
S.clavu.	-----MTVRVAVAGASGYAGGEVRLLLGHFA
M.jann.	LRITIKLSLEILLILIRIFIPKENPNKKKIGERMKEVAIGATGYTGAELRLLANHEK
S.cerev.	-----PGRVALIGARGYTKGNLVSLINGHPY
* * *	
T.thermo.	VELVGFSRRKY---EGRPLEAANPQLWDGR---LFAAQEEVLE-RAEVVFLALPNGLSM
E.coli	MNITALTVAQNDAKGLISDLHPQLKGIVDPLQPMSEISEFSPGVDVVFLATAHEVSH
Synecho.	VELTYLAGHSS---AGKPYSDLYPHLTHRVNLTIEPIDLEAIAS-RCDAVFLGLPNGLAC
Anabaena	IELVSLIAASKR-----KDAD---ERAKLLN---SVDVAILCPLDDAAR
B.subt.	AEECTLYSSSG---EGNVYSEGYPHLTGLAQQLKPIDMNTIKH-EIDIMFLAAPPGVSS
B.stearo.	VSQCDVFSSSQ---DGIHLSSEFPHVGAVDGAVLHKLEIEALA-KYDAVFFATPPGVSG
S.clavu.	ESWVALTAFSQ---AG---QAVGHPHLGPVAGRVFEPISAQAPLA-GHDVFLALPPAQA
M.jann.	VNVYITTSRKE---AGKHVFKVHPLKGIKLYKNCFTGIDDKV-DAYLVFTATPHGASM
S.cerev.	LEVAHVSSREL---KGQKLQDYTKSEIYESLQIQDIRKLEEQN-AVDFFWMALPNKVCE
* * *	
T.thermo.	EIAP--EALKAGKRVVD--LSGDFRLP-PEVYEAWYRIPKSPDLYREAVYGLPELH---
E.coli	DLAP--QFLEAGCVVFD--LSGAFRVNDATFYEKYGFTHQYPELLEQAAYGLAEWC---
Synecho.	DMAP--ALLAKGCKVLD--LSADYFRNLNTYETWYKKDRQDQATNNQAVYGLPELY---
Anabaena	EAVS--LVNSSQVKILD--ASTAYRTA-----QG-----WV-YGFPENMPGQ
B.subt.	ELTP--KLADAGITVID--LSGDLRIKEPAEYKWKRTAAPKAVIQEAVYGLAELN---
B.stearo.	EWAP--ALVDRGVKVID--LSGDFRLKDGAVYQWYGREAPSAYLRAVYGLTEWN---
S.clavu.	ALA-----EEAGARVDRNMGADFRLDAGVWERFYGSPWRG---CWP-YGLPESPG-A
M.jann.	DIVP--DFTERGMKVID--LSGDYRFEDLSLYEKYKIKHKG-LPDVKIAYGLPELH---
S.cerev.	PFVETIQSVHGKSKIID--LSADHRFV-----SE-----SDWAYGLPELN--D
* * *	
T.thermo.	REELKGARLVANPGCYVTAATLALAPLAEEVGLKGA---FVVGLSGVSGAGR-EAECTAL
E.coli	GNKLEKANLIAVPGCYPTAAQLALKPLIDADLLDLNQWVINATSGVSGAGRKAASNSF
Synecho.	RDAIRDAQLIGCPGCTPTASLLALSPLLKQGFIVPETA-IIDAKSGTSGGGREAKVNMML
Anabaena	REKIANAQFVSNPGCYPTGFLACVRLPIAQQILPSSFPITINAVSGYSGGKSLIQKYS
B.subt.	QLQIQAKLIANPGCFPTAVLLGLAPLAQKLLDESIV-IDAKTGVSGAGRKASMGTHF
B.stearo.	REAVRGAVLLSNPGCYPTATLLGLAPLVKEGLIKEDSI-IDAKSGVSGAGRKAGLGTHTF
S.clavu.	REALAGARRIAVPGCYPTAVTLALFPVAGVGLVDRE---VVTAVSGTSGAGRALKPHLLG
M.jann.	REEIKAEQLVANPGCFPTGAILAVAPLVKENIIEER-I-IFDSKTGVSGAGIKPTETTHF
S.cerev.	RAKIANAANPGCYATGSQLTISPLTKYINGLPT---VFGVSGVSGAGTKPSPKNDP
* * *	
T.thermo.	AEVVEN-----LKPVKAGOTHRHIPEMERNLGRILAQGRVRTHGEARAVRLSFTHHL
E.coli	CEVS-----LQPYGVF-THRHQPEIATHLGADV-----IETPHL
Synecho.	AEAEGS-----LGAYGVA-KHRHTPEIEQIASDLAGT-----ELRVQFTPHL
Anabaena	PHQEQGATSDYFPGIYGLQFGHKHVKEMHQHSGLAS-----PPLFIPAV
B.subt.	SELNDN-----FKIYKVN-EHQHTPEIEQALNEWPG-----LGPIITFSAHL
B.stearo.	SEVNE-----VKIYKVN-AHQHIPEIEQALQTNWNEA-----VAPITFSTHL
S.clavu.	AEVMS-----VSPYAVGGTHRHTEIAQNSTAVTDG-----EPVSVSPTPL
M.jann.	PNVNE-----INPYKIT-THRHTEIEKELKKLG-----KAKVSFTPHL
S.cerev.	KFLNN-----LIPYALS-DHIEREISARIGHNV-----AFMPHV
* * *	
T.thermo.	VPMTGRSWS-PRGRGGGR---LEPGEPRGALPGLLRGAFVRVLK-----GLPET
E.coli	GNFPRGILETITCRKLSG---VTQAQVAVQLQQAAYAHKPLVRLYD-----KGVPAL
Synecho.	IPMVRGILATVYATLRDPG---LVRDDLITISAFYRASFPVKILPH-----GVYPQT
Anabaena	GDFEQGMVLQIPLPLWTLDNPPSGEETHQAIQYQGEKFPVQVAPFKDPSLLRDGTFLDA
B.subt.	FPMTGRGIMATMYTRLTCD---LTADDLHDLYSEFYQDSYFVRVRPK-----GQYPQT
B.stearo.	IPMTGRGIMATYAKAKQS---ISPNDLVLYKTSYEGSPFVRIRQL-----GQFPAT
S.clavu.	APMPRGILATCSARLTPTG---ERRQVRAGLTCKTYADEPFVSLAA-----GGRVAL
M.jann.	APITRGLTTAHTFLAKD---VDREEIKIYEKFGSEVFRIFS-----EELPKL
S.cerev.	GQWFGQISLTSVSIPIKKS---LSIDEIRKLYRNFYEDELHVHVID-----DIPLV
* * *	
T.thermo.	KATLGSN-RVDVRPLYEERTGRVLVFAALDNLVKGMAAG---QAVQNLMMLGLPEETALPK
E.coli	KNVVGLP-FCDIGFAVQG---EHLIIIVATEDNLLKGAAA---QAVQCANIRFGYAEQTSLI-
Synecho.	KWAWGNTN-LCYIGIEVDPTGRVIVLSAIDNLVKGAG---QAVQCLNLMGMWEEESLGLPQ
Anabaena	TAVNGNTN-LVQVVFVGNQNTKEALLVARLDNLKGASG---AAVQNLMMLGLPEELGL-
B.subt.	KEVYGSN-FCDIAVTLDETRNRTIVSVIDNLMKGAAG---QAVQNFNLMGMWNEETGLTI
B.stearo.	KDVYGSN-YCDIGLAYDERTERVTVSVIDNLMKGAAG---QAVQNFNLMGMWDEAEGLRS
S.clavu.	DGFDGLR-SAHVVDVRRR---VVVKARDRNLTKGAAGRRRLQSMNLALGLPETTGLPR
M.jann.	TWVRGNTN-FCDIGFPEIDEGRLVVISALDNLVKGASG---QATQNMNMFMGFDEKEGLFD
S.cerev.	KDIEGTHGVVIGGFKLNDADRVVVCATIDNLLKGAAAT---QCLQNINLMAGYGEYAGIPE
* * *	
T.thermo.	EGLWP-
E.coli	-----
Synecho.	LAFYP-
Anabaena	-----
B.subt.	TPIYP-
B.stearo.	LPIYP-
S.clavu.	TGLAP-
M.jann.	VGLNP-
S.cerev.	NKIIGV

Fig. 3. CLUSTAL W multiple sequence alignment of nine NAGSDs. Similar amino acids are indicated by dots below the alignment and identical amino acids by asterisks. Abbreviations: T.thermo., *Thermus thermophilus*; E.coli, *Escherichia coli* (A42377); Synecho., *Synechocystis* sp. (D90912); Anabaena, *Anabaena* sp. (S24753); B.subt., *Bacillus subtilis* (I40372); B.stearo., *Bacillus stearothermophilus* (I39765 and Savchenko et al., 1996); S.clavu., *Streptomyces clavuligerus* (A92987); M.jann., *Methanococcus jannaschii* (L77117); S.cerev., *Saccharomyces cerevisiae* (Q01217).

Characteristics of *argC*

The G+C content of *T. thermophilus argC* is 69.1 mol%, which is in perfect agreement with that of the species' chromosomal DNA (69 mol%) (Nagahari et al., 1980). The codon usage is strongly biased towards the use of G or C in the first (71.8%) and third (86.7%) codon positions, the GC frequency in the second codon position (48.1%) is comparatively low. The *T. thermophilus argC* gene product shows significant similarity

(similar and identical amino acids) to those of several other micro-organisms (Table 1).

The amino acid composition of *T. thermophilus* NAGSD is listed in Table 2. There is a significant increase in two charged residues (glutamic acid and arginine) in the *T. thermophilus* enzyme, which could in principle be involved in protein-stabilizing salt bridges. This tendency was also observed in other thermostable enzymes [*B. stearothermophilus* triosephosphate iso-

Table 2. Percentage amino acid composition of nine NAGSDs

The composition of the sequences was evaluated relative to the residue usage quantile table (Brendel *et al.*, 1992). Low usage in the 5% quantile is indicated by —, and in the 1% quantile by — —; high usage above the 95% quantile point is indicated by +. Abbreviations: T.th., *Thermus thermophilus*; E.co., *Escherichia coli*; Syne., *Synechocystis* sp.; Anab., *Anabaena* sp.; B.su., *Bacillus subtilis*; B.st., *Bacillus stearothermophilus*; S.cl., *Streptomyces clavuligerus*; M.ja., *Methanococcus jannaschii*; S.ce., *Saccharomyces cerevisiae*.

	T.th. (344 aa)	E.co. (334 aa)	Syne. (351 aa)	Anab. (322 aa)	B.su. (345 aa)	B.st. (345 aa)	S.cl. (340 aa)	M.ja. (375 aa)	S.ce. (342 aa)
A	11.3	12.0	10.3	9.3	8.4	10.7	15.3 +	5.9	7.1
G	12.2 +	7.8	9.4	9.3	8.1	9.3	12.1 +	7.7	8.6
M	1.5	0.9	1.7	1.6	2.6	2.0	1.5	1.3	0.9
S	3.5 —	4.8	4.6	6.5	4.9	6.7	5.3	3.2 —	6.5
C	0.3	2.1	2.0	0.9	1.2	0.9	0.9	0.8	1.2
H	2.3	3.6	2.3	1.9	2.9	2.6	2.4	3.5	3.1
N	2.6	4.2	3.1	5.0	4.3	3.5	1.2 —	4.5	5.9
T	3.2 —	5.1	5.1	3.7	7.0	4.3	5.9	5.9	3.7
D	1.7 —	4.5	4.8	5.6	4.9	4.9	3.5	4.5	5.2
I	1.5 —	5.4	6.3	6.2	7.0	5.8	1.5 —	11.2 +	9.6 +
P	7.0	5.4	6.0	6.2	5.5	4.9	7.9	4.8	4.9
V	8.1	8.4	6.0	6.5	5.8	8.7	10.3 +	6.4	7.4
E	9.6	4.5	4.8	5.0	6.7	6.1	4.4	8.8	5.9
K	3.8	3.6	3.4	5.0	5.2	4.3	1.5 —	9.6	6.8
Q	1.5 —	5.7	4.0	6.0	4.6	3.2	2.1	0.8 —	3.4
W	1.5	0.6	1.1	0.6	0.9	1.4	1.2	0.3	0.9
F	2.3	4.2	2.0	4.7	3.2	3.2	2.6	5.1	2.8
L	14.2 +	11.4	12.8	9.9	9.6	8.7	9.7	8.5	8.6
R	9.3 +	2.4	5.1	3.1	2.9	4.1	9.1	4.0	3.4
Y	2.6	3.6	4.8	3.1	4.3	4.6	1.8	3.2	4.0
R/R + K*	0.72	0.4	0.38	0.48	0.36	0.86	0.58	0.33	0.29

* R/R + K, number of arginine residues/sum of arginine and lysine residues.

merases, (Rentier-Delrue *et al.*, 1993); *T. thermophilus* DNA ligase, (Lauer *et al.*, 1991)]. As regards hydrophobic residues, we noted a higher leucine content and a lower isoleucine content. Again, this was observed previously when comparing the *T. thermophilus* DNA ligase to its *E. coli* homologue (Lauer *et al.*, 1991).

There is only one cysteine residue in *T. thermophilus* NAGSD and it is probably involved in the reaction mechanism (see Discussion). A lower cysteine content has been reported in several thermophilic enzymes (Koyama & Furukawa, 1990). The lower content of asparagine and aspartic acid is in agreement with the results of a comparative analysis of triosephosphate isomerase (Rentier-Delrue *et al.*, 1993) but this thermophilic feature, as well as the higher proline content (Watanabe *et al.*, 1991) has to be weighted against a possible bias introduced by the high GC content (see Discussion).

Threonine, serine and glutamine are less abundant than in the other known NAGSDs except that of *Methanococcus jannaschii*, another extreme thermophile. This proved also to be the case when comparing thermostable DNA ligase to ligases from mesophilic organisms (Lauer *et al.*, 1991) and when comparing triosephosphate

isomerase of *B. stearothermophilus* with that of the psychrotroph *Moraxella* sp. TA137 (Rentier-Delrue *et al.*, 1993).

Improved protein stability with a higher Arg/Arg + Lys ratio is believed to arise through higher hydrophilicity of arginine residues (Merkler *et al.*, 1981) and through increased hydrogen bonding capability at the guanidinium group of arginine residues (Mrabet *et al.*, 1992). With the exception of *Strep. clavuligerus*, the *T. thermophilus* NAGSD has the highest Arg/Arg + Lys ratio.

The *argC* gene product has a predicted M_r of 37 600 and a calculated pI of 9.49.

Characteristics of *argJ*

The G + C content of the *argJ* gene is 70.8 mol%. A G or C is found in 69.3% of the cases in the first codon position, in 50.9% in the second and in 92.1% in the third.

The amino acid residues present in eight OATs of different origins are shown in Table 3. Again, the *T. thermophilus argJ* gene product shows trends that have been observed in many thermophilic proteins (see

Table 3. Percentage amino acid composition of eight OATs

The composition of the sequences was evaluated relative to the residue usage quantile table (Brendel *et al.*, 1992). Low usage in the 5% quantile is indicated by —, and in the 1% quantile by — —; high usage above the 95% quantile point is indicated by +, and above the 99% quantile point by + +. Abbreviations: T.th., *Thermus thermophilus*; N.go., *Neisseria gonorrhoeae*; Syne., *Synechocystis* sp.; B.su., *Bacillus subtilis*; B.st., *Bacillus stearothermophilus*; C.gl., *Corynebacterium glutamicum*; M.ja., *Methanococcus jannaschii*; S.ce., *Saccharomyces cerevisiae*.

	T.th. (381 aa)	N.go. (406 aa)	Syne. (433 aa)	B.su. (406 aa)	B.st. (410 aa)	C.gl. (388 aa)	M.ja. (402 aa)	S.ce. (441 aa)
A	17.1 + +	16.3 +	13.8 +	10.8	13.4 +	15.7 +	8.7	7.7
G	10.5	7.1	9.7	9.1	8.5	9.5	8.5	7.3
M	2.4	2.5	3.0	2.2	2.9	3.1	3.2	2.7
S	2.9 —	4.4	6.0	5.4	4.1	6.4	4.0	7.9
C	0.5	1.5	1.4	2.0	1.7	1.5	1.5	1.6
H	0.5 —	2.0	0.5 —	2.0	1.7	0.3 —	0.5 —	0.5 —
N	3.1	3.7	3.7	3.9	3.7	5.4	7.0	6.6
T	5.2	8.4	8.8	7.9	8.3	8.5	6.0	8.4
D	3.9	6.9	5.5	5.4	6.3	7.7	6.5	6.3
I	3.4	6.7	5.8	7.6	5.6	4.1	7.7	6.6
P	5.0	3.7	3.5	2.7	3.4	2.8	1.7 —	2.3
V	8.1	7.1	5.3	7.4	10.2 +	9.0	10.9 +	7.5
E	8.1	4.4	3.7	8.1	5.4	4.6	9.0	4.5
K	2.4	4.7	3.7	6.9	5.4	3.4	9.2	7.9
Q	1.8	4.2	6.2	3.9	4.6	3.1	0.5 — —	3.4
W	1.0	0.2	1.4	0.7	0.7	0.5	0.2	0.7
F	2.4	2.7	3.7	2.2	2.0	3.1	3.7	4.1
L	10.5	7.1	8.1	7.1	6.3	7.0	5.7	9.3
R	8.7	4.2	4.4	2.0 —	2.7	3.1	2.7	2.9
Y	2.4	2.2	1.8	2.5	2.9	1.0 —	2.7	1.8
R/R + K*	0.79	0.47	0.48	0.33	0.22	0.54	0.27	0.23

* R/R + K, number of arginine residues/sum of arginine and lysine residues.

above): a higher presence of arginine, leucine, proline, glycine and alanine; a markedly lower content of serine, cysteine, threonine, aspartic acid, isoleucine, lysine, glutamine (except for *M. jannaschii*) and asparagine. However, it should be remembered that Lys is only represented by two A-rich codons. As for NAGSD, the Arg/Arg + Lys ratio is highest for the *T. thermophilus* OAT.

A CLUSTAL W multiple sequence alignment of the eight known *argJ* amino acid sequences (Fig. 4) shows that the two monofunctional OAT enzymes (of *T. thermophilus* and *C. glutamicum*) are slightly shorter at the N-terminus than the OATs known to be bifunctional (*B. stearothermophilus*, *B. subtilis* and *N. gonorrhoeae*).

The calculated M_r of the *T. thermophilus* OAT is 40 300 and the theoretical pI is 5.85.

ORF1 and ORF4 nucleotide and amino acid sequences

The total G + C content of the truncated ORF1 is 66.5 mol%. The use of G or C in the first codon is 75.6%, in the second 39.7% and in the third 84%. As evidenced by the avoidance of rare codons and the high

GC content in the third position (which is comparable to that of the *argC* and *argJ* genes), ORF1 is probably part of the coding region for an as yet unidentified protein.

The G + C content of the truncated ORF4 is 60.9 mol%. The use of G or C in the first, second and third codon position is 63.6%, 43.9%, and 75.1%, respectively. These data deviate from the general rule for *Thermus* coding regions and the codon usage also deviates from what is commonly seen (Nakamura *et al.*, 1997).

Properties of OAT

Repression of OAT synthesis by arginine. Addition of 5 mM arginine to the growth medium resulted in a more than twofold repression of OAT activity [0.5 units (mg protein)⁻¹ in extract of cells grown in the presence of arginine, as compared to 1.3 units (mg protein)⁻¹ in extract of cells grown in minimal medium]. Ornithine had no repressing effect.

Feedback inhibition of OAT. In Fig. 5 the effect on OAT activity of the addition of either ornithine or arginine to the reaction mixture is shown. At saturating levels of substrates arginine had only a weak inhibitory effect

T.thermo.	-----MAVRLPRGFRAGATRAGIKPSGRPDALLVS---GLP
C.glut.	-----MAEKGITAPKGFVASATTAGIKASGNPDMALVNV---QGPE
N.gonn.	-----MAVNLTEKTAELPDIDGIALYTAQAGVKKPGHTDLTLIAV---AAG
B.subt.	-----MIQLSEDIKVKVT---GDVSSPKGFQAKGVHCHGLRYS---KKDLGVLIIS---ETP
B.stearo.	-----MTIITKQTGQVTAADGTVVTEPGFQAAGVNAAGLRYS---KNDLGVILC---DVP
Synecho.	-----MWQAFPMADWQVIEGNITAPKGFKAAGITAGLKPSGSPDLISLIYSF---TETE
S.cerev.	MRISSTLLQSKQLIDKYALVVPKTSFPGKFEVGYTAGSVKKNGLDLGVILNTNKSRR
M.jann.	-----MRVIDGGVTAPKGFKAN---GYKEG-KFGVAIIIS---EKD
T.thermo.	AAWAYATQNRAAAPSIHGRALYASGK---PLRAVVVNAGNANCATGERGYEDDRR---MA
C.glut.	FSAAAVFTNRNVFAAPVKVSRENVDG---QIRAVLYNAGNANACNGLQGEKDARES---V
N.gonn.	STVGAVFTNRFCAAPVHIKSHLFDDE---GVRALVINTGNANAGTGAQGRIDALA---VC
B.subt.	AVSAAVYTSQSHFQAAPIKVTQDSLKHGP---TLKAVIVNSAIANACTGEQGLKDAYT---MR
B.stearo.	ASAAAVYTSQSHFQAAPIKVTQASLAVEQ---KLQAVIVNRPANACTGAQGLKDAYE---MR
Synecho.	ALAAAGVFTTSQVRAACVDYCRQLQAKA---SARAILVNSQANAGTGKQGWDDAVESAF
S.cerev.	STAAAVFTTNKFAAPVLTSSKVLLETARGKNINAILVNSGCANSVTGDLGMKDAQV---MI
M.jann.	AVGAGTFTTNKVVHVPVLSRELKNRD---KFRALVANSNANCFT---KDGMEDEK---MQ
T.thermo.	ELAALRLGLSPEEVLTAAGTGVIGVPLPVEKIAKGLPEIG-----LTPFAEAFAEAILT
C.glut.	SHLAQNLGLSDIGVCSSTGLIGELLPMKDLNAGIDQLTAE-----PLGDNAAAAKAIMT
N.gonn.	AAAAAQIGCKPNQVMPFSTGVILEPLPADKIIAALPKMQ-----PAFWNEAARAIMT
B.subt.	ESFASQLGTPELVAVSSTGVIGEHLMDEKIHAGIELLK---E-----TPAGSGDFEEAILT
B.stearo.	ELCAKQFGLALHHVAVASTGVIGEYLPMEKIRAGIKQLVPGV---TMADAEAFQTAILT
Synecho.	QLLAQGLNLTSPESILLASTGVIGQRKMEQLRQGITPLIQLS---APNGDKAARAIMT
S.cerev.	DLVNDKIGQK---NSTLVMSTGVIGQRLOMDKISTGINKIFGEEKFGSDFNSWLNVAKSICT
M.jann.	RLVAELFNINEDEVLAAGTGVIGRKMMDNIIKDRINKVYNLI---KEGNSSINAAKAIMT
T.thermo.	TD---TRPKVAEAEVAG-----ARIVGLAKGSGMIHPNM---ATMLAFLVTDALVPQEAIRA
C.glut.	TD---TVDKETTVFADG-----WTVGGMKGKVGCMAPSL---ATMLVCLITDASVTQEMAQI
N.gonn.	TD---TVPKAASREGKVGQDHTVTRATGIAGSGMIHPNM---ATMLGFIATDAKVSQPVQL
B.subt.	TD---TVIKQTCYELAIGGK---TVTIGGARKGSGMIHPNM---ATMLGFVTTDAIEEKALQK
B.stearo.	TD---TVMKRACQYTTIDGK---TVTVGGAAKSGSGMIHPNM---ATMLAFITTDANVSSPVLHA
Synecho.	TD---TDLFTVPKTTIALETEIDGR---PVRMGGIAKSGSGMIHPNM---ATMLAFVTCDAVSTALWQQ
S.cerev.	TD---TFPKLVTSRFLPSGTETTLTGMAKGAGMCPNM---ATMLGFIVTDLPIESKALQK
M.jann.	TD---TKPKIEIAVEFEVNGK---TVRVGGIAKGAGMIAPNMLHATMLCFITTDIEIDKESLTN
T.thermo.	GWRRVVERT---FNQVTVDDGTSTNDLALLMANGAYG-----EVPLKAFFEGVEAEAQ
C.glut.	ALANAFVAVT---PDTLIDIGSTSTNDTVFLASGASG-----ITPTQDELNDVAVYACS
N.gonn.	MTQEIADET---FNITITVDGDTSTNDSFVLIATGKNSQSEIDNIADPRYAQLKELLCSLAL
B.subt.	ALRETTDVS---FNQITVDGDTSTNDMVLVMAACAEN---ECLTEDHDPDWPVFKKALLTCE
B.stearo.	ALRSITDVS---FNQITVDGDTSTNDMVLVMAASGLAGN---DELTFDHPDWNFYEAALRTCE
Synecho.	MLSRATQFTKTFNQVTVDDGTSTNDSLALANGESRT---AAITEMGPNAEKLEAMLTAVQC
S.cerev.	MLTFATTRS---FNCISVDGDMSTNDTICMLANGAIDTKEN---EDSKDFEQVKLQVTEFAQ
M.jann.	ILQKVVDKT---FNNISVDGDTSTNDTVFVLANGLSG-----VNYEECGEEFENALLYVCR
T.thermo.	ELARMIAED---GEGATKLMVVRVVEATEEGARRAARALAGSALWSALYGNPDNWRGRL
C.glut.	DIAAKLQAD---AEGVTTRVAVTVGTITNNEQAINAARTVARDNLKFCAMFGSDPNWGRVL
N.gonn.	ELAQAIVRD---GEGATKFIITVRVENAKTCDEARQAAYAAARSPLVKTAFFASDPNLGKRL
B.subt.	DLAKEIARD---GEGATKLI EAQVQGAKNLNDANVIAKKIVGNSLVKTAFFAVGTDANWGRII
B.stearo.	DLAKAIARD---GEGATKLI EVRVGAKTDEEAKKIAKQIVGNSLVKTAFFAVGADANWGRII
Synecho.	HLAKAIARDFTGEGATCLMEIQVTGAPDDQARAVARTAGSSVLKSAVFRDPNWRGRIA
S.cerev.	RLAQLVVRD---GEGSTKFTVTVNKNALHFEAKIIAIESISNSMLVKLTALYQDANWGRII
M.jann.	ELAKMIVKD---GEGATKFMVSVVKGAKTEEDAVKASKAIVNSLVKTAFFAVGSDPNWGRIV
T.thermo.	AALGNS-GARFDPL---RVRIVLQGIPLYEG-AVLPPFDRE-----EAGQAMRAEEV
C.glut.	AAVGMA-DADMEPE---KISVFPNGQAVCLDSTGAPGARE-----VDLSGADI
N.gonn.	AAIGYADVADLTD---LVEMYLDDILVAEHGGRAASYTEA-----QQQAVMSKDEI
B.subt.	GATGHS-AAQVTAE---EVEVYLGQCLFKNNPEQPFSES-----TAKEYLEGDEI
B.stearo.	GATGYS-DAEVPND---NVDVAIGPMVLMKSGEPQPFSEE-----EAAAYLQQETV
Synecho.	GAAGRA-GVKFFTDQN-NLLIKLGNVYVMDQGGPLEFDRFGASNYLKQAASGAYLEQDTV
S.cerev.	CATGYAKLNDLKSIDVKNVSVFIATDENSEPRELKLAVANGVPQLEIDETRASEILALNDL
M.jann.	AAVGYS-GADFPNEVV-DVILSNYKDEVYLVKDGIPLADEG---TEELKKAEEIMKSDIE
T.thermo.	EVLVDLRE-GQGAGE---AYGCDLTEDYVRINALYTT
C.glut.	DVRIDLGTSGEQAT---VRTDLSRSYVEINSAYSS
N.gonn.	TVRIKLHR-QQAAT---VVTCDLSHGYVSINADYRS
B.subt.	TVIVKMAE-GDGNR---AWGCDLTIDYIKINASYRT
B.stearo.	VIEVDLHE-GDGVGV---AWGCDLTIDYIKINASYRT
Synecho.	LIQVDLGT-GSGQGFTTAWGCDLSYDYVRINADYTT
S.cerev.	EVSVDLGT-GDQAAQ---FWTCDLSHEYVTINGDYRS
M.jann.	KIVVDLKM-GEFENV---CYGCDLSYDYVRINAEYTT

Fig. 4. CLUSTAL W multiple sequence alignment of eight OATs. The upper two enzymes are monofunctional, the *B. stearothermophilus* and *Sacch. cerevisiae* enzymes are bifunctional (enzymological data) and the *B. subtilis* and *N. gonorrhoeae* enzymes are probably bifunctional (complementation data). For the *Synechocystis* and *Methanococcus* enzymes no data are available. Similar amino acids are indicated by dots below the alignment and identical amino acids by asterisks. Abbreviations: T.thermo., *Thermus thermophilus*; C.glut., *Corynebacterium glutamicum* (X86157); N.gonn., *Neisseria gonorrhoeae* (P38434); B.subt., *Bacillus subtilis* (P36843); B.stearo., *Bacillus stearothermophilus* (Q07908); Synecho., *Synechocystis* sp. (D90912); S.cerev., *Saccharomyces cerevisiae* (M. Cabaal, personal communication); M.jann., *Methanococcus jannaschii* (L77117).

(unlikely to be physiologically significant) with an apparent K_i of 26 mM. Under the same conditions, ornithine functions as a much stronger inhibitor, the apparent K_i being 1.4 mM.

Partial purification and molecular mass determination of OAT. OAT was purified about 40-fold from a cellular extract of *T. thermophilus* (see Methods for the purification protocol). The enzyme proved to be very thermostable, retaining 100% activity after 15 min at 95 °C. An M_r of 86000 ± 8000 was obtained by gel filtration, indicating that the enzyme could be a homodimer.

DISCUSSION

We have cloned and characterized the *T. thermophilus* gene (*argJ*) encoding the enzyme which deacetylates acetylornithine in the arginine biosynthetic pathway. As in most other micro-organisms (except the *Enterobacteriaceae* and *Sulfolobus*, see Introduction), *T. thermophilus* therefore uses the energetically favourable mechanism which recycles the acetyl group of N-acetylornithine onto glutamate. The reaction has already been reported in *Thermus* ZO5 by Van de Castele *et al.* (1990). The synthesis of this OAT was found to be repressible by arginine, which is in keeping

Fig. 5. Inhibition of OAT by ornithine (▲) and arginine (●). Inhibition was tested by adding inhibitor to the reaction mixture of the enzyme assay described by Van de Casteele *et al.* (1990).

with its anabolic function. The native enzyme has an M_r of 86 000; from the value derived from the gene sequence (40 300) it would appear that the protein is a dimer, as was also postulated by Sakanyan *et al.* (1993) for *B. stearothermophilus*. In *N. gonorrhoeae*, the M_r of the *argJ* gene product was determined to be 46 000 by minicell analysis (Martin & Mulks, 1992). In *Sacch. cerevisiae*, Liu *et al.* (1995) showed the enzyme to be a heterodimer of M_r 57 000, with a small subunit of M_r 26 300 and a large one of M_r 30 700. This unexpected heterogeneity appears to result from processing of the primary translation product (Liu *et al.*, 1995).

Alignment of eight known OAT sequences (Fig. 4) showed no obvious amino acid pattern that could account for the difference between mono- (*T. thermophilus* and *C. glutamicum*) and bi- (*B. stearothermophilus*, *B. subtilis*, *N. gonorrhoeae* and *Sacch. cerevisiae*) functional OATs. The search for a possible pattern is rendered even more problematic by the unexpected lack of any apparent homology between OAT and N-acetylglutamate synthases (Sakanayan *et al.*, 1996). It is notable, however, that the two known sequences of enzymes characterized as monofunctional are somewhat shorter at the N-terminus, which may be correlated with the fact that a fragment lacking a sizeable portion (unsequenced) of *B. subtilis argJ* was reported to complement *argE* but not *argA* *E. coli* mutants (Mountain *et al.*, 1986). It could therefore be interesting to undertake a deletion analysis of bifunctional *argJ* genes.

Sequence analysis of the total 3.2 kb DNA fragment comprising the *argJ* gene demonstrated the presence of an *argC* gene immediately upstream, but no putative promoter sequence could be found 5' to *argC*. Instead, we found an unidentified ORF. It looks as if these three genetic entities are part of an operon, the more so since the stop codon of *argC* overlaps with the start codon of *argJ*. If ORF1 is functional this would be the first case of

an arginine operon including an unrelated gene. By contrast, in the *Enterobacteriaceae* and in *B. stearothermophilus*, *B. subtilis*, *Strep. coelicolor* and *Strep. clavuligerus*, *argC* is the first gene of an arginine operon. Downstream of *argJ* another unknown ORF is present. It is preceded by a putative promoter sequence, which suggests that it is part of a separate genetic entity. The genetic organization of the arginine biosynthetic genes in *T. thermophilus* thus appears to be of a new type: ORF1–*argC*–*argJ*. Elucidation of up- and downstream sequences is in progress. The possible role of ORF1 is at present entirely conjectural. Clusters of functionally related genes – some of them well characterized operons – containing apparently unrelated ORFs have been reported in other organisms (e.g. see Fani *et al.*, 1995). One hypothesis envisages that such ORFs may code for proteins which contribute to the stabilization of enzymes encoded by the same polycistronic mRNA (Glansdorff, 1997).

Recently Tabata & Hoshino (1996) mentioned using an *argCB* clone (isolated in their laboratory) in physical mapping of the *T. thermophilus* HB27 genome. The cognate *argCB* fragment was later described to encode the *argC* gene (ORF of 935 bp, 94.6% G or C in the third codon position) upstream of an *argB* gene (ORF of 809 bp) (Kosuge & Hoshino, 1996). Both the length of the *argC* encoding the ORF and the GC content in the third codon position markedly differ from those described in the present paper (ORF of 1032 bp, 86.7% G or C in the third codon position). Moreover, the gene sequence *argCB* (Tabata & Hoshino, 1996) differs from *argCJ* (found in our laboratory). A possible explanation for this contradictory situation could be that *argC* has been duplicated in the genome of *T. thermophilus* HB27 and that the two copies have undergone mutational divergence over a long period of time. We checked this hypothesis by Southern hybridization using a *Bam*HI *argC* gene fragment (isolated from the 3233 bp fragment, see Results) as a probe against *Bam*HI-digested *T. thermophilus* genomic DNA under low stringency conditions (data not shown). However we detected only one hybridizing band on the X-ray film. This leaves the question of two different *argC* copies on the *T. thermophilus* HB27 genome open and unresolved. Publication of the *argCB* gene sequence by the Japanese group might help to clarify the situation.

Overall sequence comparisons between the large number of mesophilic and thermophilic proteins which have now been reported do not point to universal 'traffic rules' that would allow firm predictions regarding the molecular basis of thermophily (Daniel *et al.*, 1996). This is due in part to the fact that different molecular strategies may underlie thermophily, but also to the way in which some of the comparisons were made; comparing too distant proteins (even homologous ones) may blur any tendency that could be revealed by comparing the more similar ones within a family of homologous proteins. In the present case, if *T. thermophilus* OAT (a very stable enzyme) is compared with its homologue from *Synechocystis* (47.3% identity), the trends already

observed in several other proteins (see Results) assert themselves quite clearly: a lower isoleucine, serine, cysteine, asparagine, threonine and aspartic acid content; and a higher leucine, proline, glutamic acid and arginine content (also a higher Arg/Arg+Lys ratio). The archaeal and presumably thermophilic OAT (from *M. jannashii*) appears quite different in these respects. The same remarks hold for a comparison between *T. thermophilus* NAGSD and its homologue from *B. stearothermophilus* (45.5% identity, its host not growing at the optimal temperature of *T. thermophilus*), and the trend remains consistent when extending the comparison to other organisms (including a mesophilic *Bacillus*), except for the archaeon which again appears quite distinct, and *Strep. clavuligerus*, for which some of the trends vanish. This is not surprising since in organisms with a relatively high G+C content, GC-rich codons (e.g. Arg, Pro, Ala, Gly) may be expected to occur with a higher frequency than GC-poor codons (e.g. Asn, Lys, Tyr, Phe, Ile). For example, we find a high proline, glycine and arginine content in *Strep. clavuligerus* NAGSD (the G+C content of this bacterium is even higher than in *Thermus*, 73 mol% versus 69 mol%), although this organism is a mesophile. No three-dimensional structure of a NAGSD or OAT is as yet available to put the above considerations to the test and identify which of the observed trends is really significant.

The *argC* protein uses NADPH as cofactor. It is therefore interesting to compare the nine sequences now available among themselves [more limited comparisons were done by Ludovice *et al.* (1992) and Floriano *et al.* (1992), see below] and to other NADP-specific oxidoreductases. NADP-binding sites have been extensively studied, in particular in glutathione reductase whose three-dimensional structure is known in detail and where coenzyme specificity has been analysed by site-directed mutagenesis (Scrutton *et al.*, 1990). The site primarily responsible for binding NADPH displays a characteristic $\beta\alpha\beta$ dinucleotide-binding motif (see Wierenga *et al.*, 1985; Hanukoglu & Gutfinger, 1989), GXGX₂G/AX₃AX₆G; the G/A symbol means that the A can be replaced by a G without any dramatic effect on the kinetic parameters of glutathione reductase for NADPH (Scrutton *et al.*, 1990). When comparing the C-terminal ends of the nine *argC* proteins (Fig. 3), a strongly conserved region emerges of structure DNLXKGX₂GX₃QX₂NX₃G, reminiscent of the above motif by the relative positions of the three G residues. However, the *Streptomyces* sequence departs from this design by the inclusion of two residues. Two other strongly conserved regions of consensus GXXGXXG and GXSGXG are found in *argC* proteins, respectively close to the N-terminus (residues 5–11 in the *T. thermophilus* sequence) and towards the middle of the sequence (residues 175–180). As already noted in a previous comparison of five *argC* enzymes by Floriano *et al.* (1992), the latter sequence resembles the phosphate-binding loop of several ATP- or GTP-binding proteins. On the other hand, the N-terminal motif is

present between a predicted β sheet motif and a putative α helix that could be part of a $\beta\alpha\beta$ fold (data not shown) and the N-terminal position is reminiscent of that of several known FAD-binding sites (Hanukoglu & Gutfinger, 1989). The presence of more than one, strongly conserved putative motifs for dinucleotide cofactor binding is characteristic of FAD-containing flavoenzymes. Structural studies and mutational analysis are required to conclude which of these sequences are functional in NAGSD but, from the above comparisons, it would not be surprising if it turned out to be a FAD-containing dehydrogenase.

For two other dehydrogenases catalysing the reduction of a phosphorylated carboxyl group to an aldehyde [aspartate semialdehyde dehydrogenase (Haziza *et al.*, 1982) and phosphoglyceraldehyde dehydrogenase (Rossman *et al.*, 1975)], a cysteine is involved in the reaction mechanism. When comparing the three NAGSD sequences known at the time, Parsot *et al.* (1988) observed a strictly conserved cysteine and tentatively assigned this residue to the active site of the enzyme. In the present alignment of nine NAGSD sequences (Fig. 3), this cysteine residue remains conserved (at position 141 in *T. thermophilus*) as well as several other residues in its vicinity: AX₅PGCX₂TX₇P. Besides, in *Thermus*, this is the only cysteine found in the protein. This result therefore strongly supports the suggestion of Parsot *et al.* (1988) of a role of this cysteine in the reaction mechanism.

In the absence of structural information, the strongly conserved P-loop motif found from residue 175 to residue 180 (see previous section) appears as a possible candidate for recognition of the phosphoryl group of N-acetylglutamylphosphate.

In the cyclic pathway of ornithine synthesis, OAT may play the role of key enzymic step determining the flux of arginine precursors instead of acetylglutamate kinase, as shown for the first time in *Thermus* ZO5 where arginine was found indeed to inhibit the activity of that enzyme (Van de Casteele *et al.*, 1990). In *T. thermophilus* we found arginine to be only a weak inhibitor of OAT; ornithine however inhibits OAT with an apparent inhibition constant of 1.4 mM. This is still a relatively high value but it must be taken into account that it was determined at saturating concentrations of substrates and in crude extracts. Since *Thermus* ZO5, a closely related species, makes an inducible arginase converting arginine into ornithine and urea (C. Legrain, unpublished), *T. thermophilus* is likely to synthesize this enzyme as well; ornithine might therefore be able to curtail the flow of arginine precursors if its intracellular concentration can reach a high enough value. A similar situation was reported in *B. stearothermophilus*, where the inhibition of OAT by ornithine is however stronger (Sakanyan *et al.*, 1993).

Cloning of up- and downstream sequences relative to the ORF1–*argC*–*argJ*–ORF4 comprising 3233 bp frag-

ment is in progress. It is expected to reveal more information on the organization of arginine biosynthetic genes in *T. thermophilus* and on their regulation.

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