A re-examination of the pathway for ornithine biosynthesis in a thermophilic and two mesophilic *Bacillus* species

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The expression of *Bacillus stearothermophilus* genes complementing arginine auxotrophs of *Escherichia coli* was studied. The activity responsible for the formation of ornithine in *B. stearothermophilus* was identified as a repressible ornithine acetyltransferase (genetic symbol *argJ*) encoded by the same DNA fragment as the *argC*, *argA* and *argB* genes. *Bacillus subtilis* and *Bacillus licheniformis* displayed the same pattern of enzyme activities as *B. stearothermophilus*. In contrast to previous reports, these organisms consequently use the cyclic pathway of ornithine biosynthesis. *B. stearothermophilus* also possesses a broad specificity aminoacylase which exhibits low affinity towards *N*²-acetyl-L-ornithine.

Introduction

In micro-organisms, ornithine (a precursor of arginine) is synthesized from glutamate via *N*-acetylated intermediates (Fig. 1). The deacylation of acetylornithine can be achieved in two different ways: a linear pathway in which the formation of ornithine is mediated by the hydrolytic enzyme acetylornithinase (Fig. 1, step 5), or a cyclic pathway in which the acetyl group is recycled by transacetylation (Fig. 1, step 5'). The linear pathway is characteristic of the *Enterobacteriaceae* (Vogel & Bonner, 1956). It has also been found in the thermophilic archaeobacterium *Sulfolobus solfataricus* (Van de Casteele *et al.*, 1990). The same linear pathway was supposed to be used by the Gram-positive *Bacillaceae* (Vogel & Vogel, 1963; Udaka, 1966). Other prokaryotes, including several methanogenic archaeobacteria (Meile & Leisinger, 1984; Van de Casteele *et al.*, 1990), and all eukaryotic microbes (Davis, 1986) have been found to recycle the acetyl group by transacetylation of *N*-acetylornithine and glutamate. The expression in *Escherichia coli* of several arginine genes from mesophilic heterologous hosts has been reported (Mountain *et al.*, 1984; Jeenes *et al.*, 1986; Picard & Dillon, 1989). Complementation of *E. coli* auxotrophs by DNA from the thermophilic Gram-positive bacterium *Bacillus stearothermophilus* was reported recently (Sakanyan *et al.*, 1990).

In this work we have analysed the expression of *arg* genes from *B. stearothermophilus* in *E. coli* K12. The results led us to re-examine the status of the ornithine biosynthetic pathway in bacilli.

Methods

Bacterial strains and plasmids. These are listed in Table 1. The RecA derivative of *E. coli* K12 strain XS1D2 was obtained by selection of Tc^r UV-sensitive recombinants after conjugation with the donor strain *E. coli* K12 NK 6659. The *argE* derivative of XA4 was obtained by transduction with phage P1 grown on strain BW6165 and selection for Tc resistance.

Media and growth conditions. *E. coli* strains were grown at 37 °C in L-broth medium and in synthetic M9 (Maniatis *et al.*, 1982) or 132 medium (Falmagne *et al.*, 1965). Concentrations of antibiotics (in µg ml⁻¹) were: ampicillin (Ap), 100, and tetracycline hydrochloride (Tc), 30. *B. stearothermophilus* was grown at 55 °C in L-broth medium or in the defined AUF medium (Piérard & Wiame, 1964) supplemented with 25 mM-glucose, 5 mM-(NH₄)₂SO₄ and 0.1% (v/v) Nitsch's trace element solution (Ramaley & Hixson, 1970). *B. subtilis* and *B. licheniformis* were grown at 37 °C in liquid mineral medium 154 (Stalon *et al.*, 1967) supplemented with 25 mM-glucose and 5 mM-(NH₄)₂SO₄.

Transformation of bacteria. *E. coli* cells were grown to mid-exponential phase and made competent for transformation by standard CaCl₂ treatment (Dagert & Ehrlich, 1979).

DNA isolation and restriction. Isolation of plasmid DNA was carried out by the method of Birnboim & Doly (1979). Plasmid DNAs were manipulated according to classical protocols (Maniatis *et al.*, 1982).

Fig. 1. Arginine biosynthesis. 1, *N*-Acetylglutamate synthase (acetyl-CoA:L-glutamate *N*-acetyltransferase; EC 2.3.1.1); 2, *N*-acetylglutamate 5-phosphotransferase (ATP:*N*-acetyl-L-glutamate 5-phosphotransferase; EC 2.7.2.8); 3, *N*-acetylglutamate 5-semialdehyde dehydrogenase [*N*-acetyl-L-glutamate 5-semialdehyde:NADP⁺ oxidoreductase (phosphorylating); EC 1.2.1.38]; 4, *N*²-acetylornithine 5-aminotransferase (*N*²-acetyl-L-ornithine:2-oxoglutarate aminotransferase; EC 2.6.1.11); 5, acetylornithinase (*N*²-acetyl-L-ornithine amidohydrolase; EC 3.5.1.16); 5', ornithine acetyltransferase (*N*²-acetyl-L-ornithine:L-glutamate *N*-acetyltransferase; EC 2.3.1.35); 6, ornithine carbamoyltransferase (carbamoylphosphate:L-ornithine carbamoyltransferase; EC 2.1.3.3); 7, argininosuccinate synthetase [L-citrulline:L-aspartate ligase (AMP-forming); EC 6.3.4.5]; 8, argininosuccinase (L-argininosuccinate arginine-lyase; EC 4.3.2.1).

Restriction enzymes were obtained from the Scientific Industrial Association (Vilnius, Lithuania) or from Pharmacia, and used according to the manufacturer's recommendations.

Enzyme assays. Cells from exponential phase cultures (about 6×10^8 cells ml⁻¹) were harvested by centrifugation (10 min, 7000 g) and washed in 0.9% NaCl. Cells were suspended in 10 mM-potassium

phosphate buffer (pH 6.5) containing 15% (v/v) glycerol, 1 mM-EDTA, 1 mM-dithiothreitol and 2 mM-phenylmethylsulphonyl fluoride for *N*-acetylglutamate synthase and ornithine acetyltransferase assays or in 20 mM-potassium phosphate buffer (pH 7.5) for the other assays. Cells were disrupted by sonication for 10 min. The resulting extracts were centrifuged (15 min, 20000 g) and passed through Sephadex G-25 columns equilibrated with extraction buffer. All these operations were done below 10 °C. *B. stearrowthermophilus* enzymes were assayed at 55 °C, *E. coli*, *B. subtilis* and *B. licheniformis* enzymes at 37 °C.

N-Acetylglutamate synthase and ornithine acetyltransferase were assayed as described in Van de Castele *et al.* (1990), except that 15% (v/v) glycerol, 10 mM-MgCl₂ and 7 mM-aminooxyacetic acid were added to the incubation mixture. Aminooxyacetic acid was added in order to reduce the transamination of glutamate in crude extracts.

N-Acetylglutamate 5-phosphotransferase was measured by the ferric chloride method according to Van de Castele *et al.* (1990).

Acetylornithinase was measured by the method of Vogel & McLellan (1970).

β -Lactamase activity was determined by a microiodometric assay as described by Ross & O'Callaghan (1975).

One enzyme unit is defined as the amount of enzyme that converts 1 μ mol substrate to product h⁻¹. Protein concentrations were determined by the Lowry method.

The apparent *K_m* values for *N*²-acetyl-L-ornithine of ornithine acetyltransferase and acetylornithinase were determined using Lineweaver-Burk and Hanes-Woolf plots.

Results

Cloning of *B. stearrowthermophilus* *arg* genes

In a previous communication, Sakanyan *et al.* (1990) showed that a 3.4 kb *Eco*RI fragment of *B. stearrowthermophilus* DNA (strain NCIB 8224), when inserted in either orientation in plasmid pBR322, complemented *E. coli* mutants deficient in any of the following enzymes: *N*-acetylglutamate synthase (encoded by *argA*), *N*-acetylglutamate 5-phosphotransferase (encoded by *argB*) or acetylornithinase (encoded by *argE*). From the complementation pattern of various subfragments of the cloned DNA generated by partial *Sau*3A digestion of the parent plasmid, the order of the functional units (using *E. coli* symbols) was shown to be A-E-B (*ibid.*).

To locate the genes more precisely on the 3.4 kb fragment we proceeded to the subcloning experiments summarized in Fig. 2. Using the *Pst*I site present 0.5 kb from the proximal end of the 3.4 kb *Eco*RI fragment cloned in plasmid pAVK1 (Sakanyan *et al.*, 1990) and the pBR322-borne *Pst*I site, we constructed plasmid pAVK4 which was found to still complement *argA*, *argB* and *argE* *E. coli* auxotrophs. In a second stage, fragments obtained by the simultaneous restriction of pAVK4 by *Pst*I and *Pvu*II were ligated to plasmid pUC8 DNA digested by *Pst*I and *Hind*III, and the ligation products were used to select Arg⁺ Ap^r transformants of *E. coli* *argE* mutant MA102. The structure of one such product, pAVK7, is shown in Fig. 2. This plasmid still comple-

Table 1. *Bacterial strains and plasmids*

Strain or plasmid	Relevant genotype or phenotype	Source or reference*
<i>E. coli</i> K12		
MA101	F ⁻ <i>argA met supE supD lacZ hsdR hsdM</i>	} M. Astvatsaturyan ^a
MA102	F ⁻ <i>argE thr leu pro his thi recA rpsL ara</i>	
XS1D2	F ⁻ Δ 101(<i>ppc-argE</i>) <i>nalA rpoB</i> λ^- <i>hsdR</i>	S. Baumberg ^b
XS1D2 <i>recA</i>	as XS1D2, but <i>recA</i>	This work
XB25	F ⁻ <i>argB nalA</i> λ^- λ^s <i>hsdR</i>	} S. Baumberg ^b
XA4	F ⁻ <i>argA nalA</i> λ^- λ^s <i>hsdR</i>	
XA4 <i>argE</i>	as XA4, but <i>argE86::Tn10</i>	This work
BW6165	<i>ara41 lacY1</i> λ^{ind-} <i>xyl7 mtl2</i> <i>argE86::Tn10</i>	Wanner (1986)
NK6659	Hfr K116 <i>his thr relA rpsL srl::Tn10</i> <i>recA</i>	Laboratory collection
<i>Bacillus</i>		
<i>B. stearothermophilus</i> NCIB 8224	Prototroph	All Union Collection of Microorganisms, Moscow, USSR
<i>B. subtilis</i> C4	Prototroph	I. C. Gunsalus ^c
<i>B. licheniformis</i> ATCC 14580	Prototroph	American Type Culture Collection
Plasmid		
pAVK1	Ap ^r Tc ^r	} Sakanyan <i>et al.</i> (1990)
pAVK4	Tc ^r	
pAVK7	Ap ^r	} This work
pUC8	Ap ^r	
		Norrander <i>et al.</i> (1983)

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ments *argA* and *argE* *E. coli* auxotrophs, but no longer complements *argB* mutants. The *argA/argE* complementing activity is therefore encoded by the 2.0 kb chromosomal fragment carried by pAVK7.

We could also show that both pAVK1 and pAVK7 are able to efficiently complement the deficiencies of *E. coli* XA4 *argE*, a double *argA argE* mutant (see generation times of the complemented strains in Table 2).

Expression of cloned *B. stearothermophilus* genes

Preliminary experiments showed that the acetylornithinase activity of *E. coli* MA102 (carrying a point mutation in the *argE* gene) transformed into an arginine prototroph by plasmid pAVK1 did not exceed the background level (Sakanyan *et al.*, 1990). This suggested either that pAVK1 encodes an acetylornithinase which is highly unstable in its new cytoplasmic environment, or that the cloned gene of *B. stearothermophilus* codes for an alternative ornithine-forming function, e.g. an ornithine acetyltransferase activity. In order to distinguish between these possibilities, both acetylornithinase and ornithine acetyltransferase activities were assayed. Cell extracts of *E. coli* XS1D2 (deleted for the *argE* gene) carrying pAVK1 or pAVK7 were used. Ornithine acetyltransferase activity was detected in both these two strains, whereas no acetylornithinase could be measured. The ornithine acetyltransferase activities given in Table

2 were measured at 55 °C (optimal growth temperature of the *B. stearothermophilus* strain used in this work). When assayed at 37 °C this activity was about threefold lower. Addition of arginine to the growth medium did not significantly affect the activity. The β -lactamase levels were similar in these conditions (Table 2) as expected for similar plasmid copy numbers. The apparent K_m value of ornithine acetyltransferase for *N*²-acetyl-L-ornithine at 10 mM-glutamate was 0.3 mM (measured on dialysed crude extract of strain XS1D2 carrying the pAVK7 plasmid).

In an *E. coli argA argE* double mutant (strain XA4*argE*) transformed to arginine prototrophy by pAVK1 or pAVK7 DNA, both the *N*-acetylglutamate synthase and the ornithine acetyltransferase activities encoded by the *B. stearothermophilus* genes were expressed (Table 2). However, addition of arginine to the culture medium led to a drastic decrease of enzyme levels in strain XA4*argE* carrying pAVK7. The very low level of β -lactamase in this culture (Table 2) indicates that plasmid pAVK7 becomes very unstable in *E. coli* XA4*argE* when the only selective pressure applied is resistance to ampicillin.

Analysis of enzyme activities in *B. stearothermophilus*

The above results suggest that the cloned *B. stearothermophilus* gene that complements *E. coli argE* mutations

Fig. 2. Cloning of *B. stearotheophilus* *arg* genes. The recombinant plasmids contain chromosomal DNA from *B. stearotheophilus* strain NCIB 8224 (heavy lines) inserted into the vector pBR322 (thin lines) for plasmids pAVK1 and pAVK4 and into the vector pUC8 (thin lines) for plasmid pAVK7. The map shows the locations of the *arg* genes and the direction of transcription for *argC* (the plasmids contain only part of this gene) and *argB*, as deduced from the sequence homology with *E. coli argC* and *argB* genes (unpublished results).

codes for an ornithine acetyltransferase rather than an acetylornithinase. It was therefore of interest to examine the pathway for ornithine synthesis in *B. stearotheophilus*. *N*-Acetylglutamate synthase, *N*-acetyl 5-phosphotransferase and ornithine acetyltransferase activities were detected in extracts of *B. stearotheophilus* (Table 2). *N*-Acetyl 5-phosphotransferase and ornithine acetyltransferase synthesis were repressed by arginine [specific activity of *N*-acetyl 5-phosphotransferase $0.3 \mu\text{mol product h}^{-1} (\text{mg protein})^{-1}$ in the absence of arginine and 0.07 in the presence of arginine]. The level of *N*-acetylglutamate synthase was not affected by arginine addition. 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

ornithine acetyltransferase, but this inhibition was in both cases limited to 40% . The arginine concentrations for 40% inhibition were, respectively, 2.5 and 4 mM . The apparent K_m value of ornithine acetyltransferase for *N*²-acetyl-L-ornithine at 10 mM -glutamate was 0.4 mM . This value corresponds closely to the K_m value determined for the pAVK7-encoded enzyme. An aminoacylase displaying some activity towards *N*²-acetyl-L-ornithine was also found in *B. stearotheophilus* extracts (Table 2). However, the affinity of this enzyme for *N*²-acetyl-L-ornithine was very low ($K_m > 60 \text{ mM}$). Moreover, arginine in the culture medium did not affect the level of this activity. This aminoacylase was active towards a broad range of acyl-L-amino acid derivatives; its activity with *N*²-acetyl-L-ornithine as substrate proved much lower than with other acyl derivatives (data not shown).

Table 2. Specific activities of *N*-acetylglutamate synthase, ornithine acetyltransferase and acetylornithinase in *E. coli* strains carrying *B. stearothermophilus* genes and in *Bacillus* species

Specific activities are 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 acetylornithinase activity. β -Lactamase was assayed as a plasmid marker. ND, Not determined.

Strain	Arginine addition to growth medium*	Generation time (min)	Enzyme activity [units (mg protein) ⁻¹]			
			<i>N</i> -Acetylglutamate synthase	Ornithine acetyltransferase	Acetyl-ornithinase	β -Lactamase
<i>E. coli</i> K12 XS1D2	+	80	ND	<0.001	<0.005	<0.01
<i>E. coli</i> K12	—	100	ND	0.97 $\pm$ 0.08	<0.005	532 $\pm$ 25
XS1D2(pAVK1)	+	90	ND	0.99 $\pm$ 0.07	<0.005	539 $\pm$ 18
<i>E. coli</i> K12	—	100	ND	1.77 $\pm$ 0.15	<0.005	653 $\pm$ 24
XS1D2(pAVK7)	+	100	ND	1.20 $\pm$ 0.10	<0.005	633 $\pm$ 21
<i>E. coli</i> K12 XA4argE	+	70	<0.001	<0.001	<0.005	ND
<i>E. coli</i> K12	—	87	0.32 $\pm$ 0.04	0.71 $\pm$ 0.07	<0.005	449 $\pm$ 22
XA4argE(pAVK1)	+	83	0.30 $\pm$ 0.04	0.67 $\pm$ 0.07	<0.005	400 $\pm$ 17
<i>E. coli</i> K12	—	154	0.84 $\pm$ 0.05	2.04 $\pm$ 0.18	<0.005	1968 $\pm$ 36
XA4argE(pAVK7)	+	72	0.07 $\pm$ 0.01	0.10 $\pm$ 0.01	<0.005	32 $\pm$ 4
<i>B. stearothermophilus</i>	—	160	0.04 $\pm$ 0.01	0.28 $\pm$ 0.02	0.12 $\pm$ 0.02	ND
NCIB 8224	+	160	0.04 $\pm$ 0.01	0.07 $\pm$ 0.005	0.11 $\pm$ 0.02	ND
<i>B. subtilis</i>	—	51	0.04 $\pm$ 0.01	0.05 $\pm$ 0.005	<0.005	ND
C4	+	54	0.01 $\pm$ 0.002	<0.005	<0.005	ND
<i>B. licheniformis</i>	—	45	0.04 $\pm$ 0.01	0.03 $\pm$ 0.005	<0.005	ND
ATCC 14580	+	42	0.01 $\pm$ 0.002	<0.005	<0.005	ND

* Arginine was added at a concentration of 5 mM. Succinate (0.5%, w/v) was added for *E. coli* XS1D2 strains. *E. coli* strains carrying plasmid pAVK1 or pAVK7 were grown in the presence of Ap.

Acetylornithine deacylation in other *Bacillus* species

It has been reported previously that the formation of ornithine from *N*²-acetyl-L-ornithine in *B. subtilis* is mediated by the hydrolytic enzyme acetylornithinase (Vogel & Vogel, 1963). In the light of the results obtained for *B. stearothermophilus*, the acetylornithine deacylation step was examined in two mesophilic *Bacillus* species, *B. licheniformis* and *B. subtilis*. No acetylornithinase activity could be detected in the two strains investigated (Table 2). However, a low but significant ornithine acetyltransferase activity could be measured. Addition of arginine to the growth medium led to a strong decrease of both ornithine acetyltransferase and *N*-acetylglutamate synthase activities (Table 2), indicating a biosynthetic function. The apparent *K_m* value of ornithine acetyltransferase for *N*²-acetyl-L-ornithine at 10 mM-glutamate was 0.1 mM for the *B. subtilis* enzyme and 1 mM for that of *B. licheniformis*.

Discussion

The enzyme assays performed with extracts prepared from *E. coli* *argE* mutants (defective in acetylornithinase activity) carrying plasmid pAVK1 or pAVK7 disclosed a high ornithine acetyltransferase activity, but no acetylornithinase activity.

This provides evidence that an ornithine acetyltransferase encoded by a *B. stearothermophilus* gene can replace acetylornithinase in *E. coli*. The same observation was already reported for the *argJ* gene of *Neisseria gonorrhoeae* (Picard & Dillon, 1989). The involvement of an ornithine acetyltransferase in the arginine biosynthetic pathway of a *Bacillus* strain was, however, unexpected, since *Bacillaceae* have been reported to use the linear pathway proceeding via the hydrolytic enzyme acetylornithinase (Vogel & Vogel, 1963; Udaka, 1966). Re-examination of the acetylornithine deacylation step in two mesophilic *Bacillus* species, *B. licheniformis* and *B. subtilis*, led us to the conclusion that these organisms too recycle the acetyl group from *N*²-acetyl-L-ornithine to glutamate. The gene encoding this enzyme in these three *Bacillus* species (and presumably other ones as well) should thus be referred to as *argJ* rather than *argE*.

The acetylornithinase activity previously reported for a *B. subtilis* strain (Vogel & Vogel, 1963) could result from the action of a broad substrate specificity aminoacylase or peptidase such as those described by Cho *et al.* (1987, 1988). Such an aspecific acetylornithine deacylase activity was previously reported in yeast (Degryse, 1974) and in two strains of extreme thermophilic bacteria, *Thermus aquaticus* and *Thermotoga maritima*, which synthesize arginine by the cyclic pathway (Van de

Castele *et al.*, 1990). The *B. stearothermophilus* strain used in this work also possesses an aminoacylase activity. The gene encoding this enzyme has been cloned into *E. coli* by complementation of an *argE* deficiency (unpublished results).

The cloned *arg* genes of *B. stearothermophilus* are arranged in the same order as in *B. subtilis*, i.e. *arg C-A-J-B* (Mountain *et al.*, 1986; Sakanyan *et al.*, 1990). Partial sequencing of the bacterial DNA carried by plasmid pAVK1 indeed revealed the presence of a sequence homologous to the carboxy-terminal part of the *E. coli argC* gene at the proximal end and of a complete *argB* gene at the distal end (unpublished results). From these data it appears that the *argA/argE* complementing activity is encoded by no more than 1350 bp located between *argC* and *argB*. The size of this fragment is about the same as that determined for the *E. coli argA* gene (1332 bp; Brown *et al.*, 1987). One may thus ask whether the products of the *B. stearothermophilus argA* and *argJ* genes are two distinct proteins as in *Pseudomonas* (Haas *et al.*, 1977) or if both activities are the property of a single enzyme as suggested for the alga *Chlorella vulgaris* (Morris & Thompson, 1975) and for *Neisseria gonorrhoeae* (Picard & Dillon, 1989). A detailed functional analysis of the relevant sequence will be needed to answer this question.

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