

Arginine boxes and the *argR* gene in *Streptomyces clavuligerus*: evidence for a clear regulation of the arginine pathway

Antonio Rodríguez-García,¹ Madalena Ludovice,^{1†}
Juan F. Martín^{1,2} and Paloma Liras^{1,2*}

¹Area of Microbiology, Faculty of Biology, University of León, 24071 León, Spain.

²Institute of Biotechnology, INBIOTEC, 24006 León, Spain.

Summary

The *argR* gene of *Streptomyces clavuligerus* has been located in the upstream region of *argG*. It encodes a protein of 160 amino acids with a deduced M_r of 17 117 for the monomer. Transformants containing the amplified *argR* gene showed lower activity (50%) of the biosynthetic ornithine carbamoyltransferase (OTC) activity and higher levels (380%) of the catabolic ornithine aminotransferase (OAT) activity than control strains. Amplification of an arginine (ARG) box-containing sequence results in a 2- to 2.5-fold derepression of ornithine acetyltransferase and OTC, suggesting that the repressor is titrated out. Footprinting experiments using the pure homologous arginine repressor (AhrC) of *B. subtilis* showed a protected 38 nt region (ARG box) in the coding strand upstream of *argC*. The protected region contained two tandemly repeated imperfect palindromic 18-nt ARG boxes. The repressor–operator interaction was confirmed by band-shift experiments of the DNA fragment containing the protected region. By computer analysis of the *Streptomyces* sequences available in the databases, a consensus ARG box has been deduced for the genus *Streptomyces*. This is the first example of a clear regulation of an amino acid biosynthetic pathway in *Streptomyces* species, challenging the belief that actinomycetes do not have a well-developed regulatory system of these pathways.

Introduction

The arginine biosynthesis pathway is strongly regulated

by feedback repression in Gram-negative and Gram-positive bacteria. The *E. coli* arginine repressor (ArgR) is a 98-kDa hexameric protein that represses all the genes of the arginine regulon in the presence of L-arginine, including expression of the *argR* gene itself (Maas, 1994). ArgR binds to 18 bp consensus sequences (ARG boxes) located in the promoter regions of the arginine biosynthesis genes. ARG boxes in *E. coli* (ANTGAATAATTATTCANT) have dyad symmetry and are usually present in two copies separated by 3 bp at the control regions of arginine-regulated genes, overlapping the promoter (Charlier *et al.*, 1992). In *Bacillus subtilis*, the upstream region of the *argC* gene contains an arginine-repressible promoter and a sequence with good similarity to the ARG box operators of *E. coli*. The *ahrC* gene of *B. subtilis* encodes a protein with 27% amino acid identity to the ArgR protein of *E. coli* (North *et al.*, 1989).

Until recently, it was believed that *Streptomyces* species show little control of gene expression in amino acid biosynthetic pathways, and it has been proposed that they do not have a well-developed regulatory apparatus for such pathways (Hood *et al.*, 1992). In *Streptomyces clavuligerus* and *Streptomyces coelicolor*, arginine represses the arginine biosynthesis enzymes, but this effect appears to be not as drastic as in *E. coli*, with a 'repression ratio' of 2 to 5 in wild-type strains (Ludovice *et al.*, 1992; Soutar and Baumberg, 1996), whereas in enterobacteria and *B. subtilis* repression ratios of 10 to 50 or higher are commonplace. The 5' upstream regions of the *argC* genes in both *Streptomyces clavuligerus* (Ludovice *et al.*, 1992) and *Streptomyces coelicolor* (Hindle *et al.*, 1994) contain sequences with similarity to the *E. coli* ARG boxes. It was therefore of great interest to study whether expression of arginine biosynthesis genes in *Streptomyces* is controlled by a protein similar to ArgR and AhrC.

In this article, we report the characterization of the *argR* gene of *S. clavuligerus*, which encodes an arginine repressor, and provide evidence showing that pure AhrC repressor interacts with an ARG box of *S. clavuligerus*. These results are of great interest, since they are the first example of proper control of an amino acid biosynthetic pathway in actinomycetes challenging the 'received wisdom' that actinomycetes do not control these pathways.

Received 18 February, 1997; revised 12 May, 1997; accepted 15 May, 1997. †Present address: Instituto de Tecnologia Química e Biológica da Universidade Nova de Lisboa (ITQB/UNL), Rua da Quinta Grande 6, Apartado 127, 2780 Oeiras, Portugal. *For correspondence. E-mail degplp@unileon.es; Tel. (87) 291504; Fax (87) 291506.

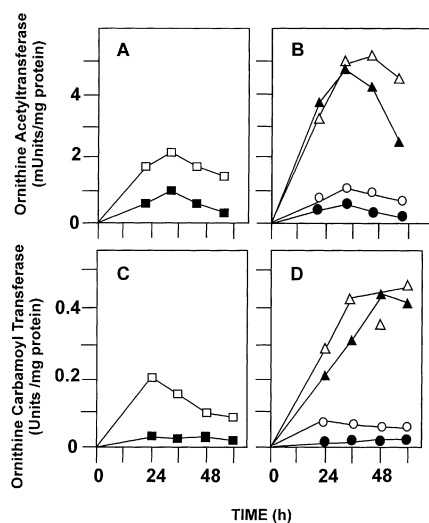


Fig. 1. Effect of arginine on the arginine biosynthetic enzymes, ornithine acetyltransferase (A and B) and ornithine carbamoyltransferase (C and D) in *S. lividans* 1326 (□, ■), *S. lividans* pIJ699 (○, ●) and *S. lividans* [pULML45] (△, ▲). Closed symbols correspond to cultures supplemented with 25 mM arginine.

Results

Effect of increased dosage of an *S. clavuligerus* ARG box on arginine biosynthetic enzymes

An AT-rich sequence resembling the arginine regulatory box of *E. coli* and *B. subtilis* is present in the upstream region of the *argC* gene of *S. clavuligerus* (Ludovice *et al.*, 1992). To elucidate the role of this putative ARG box sequence on the control of the arginine pathway, a 480 bp *Sau3A* DNA fragment containing the ARG box was subcloned in the *Bgl*III site of pIJ699, giving rise to plasmid pULML45. This plasmid was transformed into both *S. lividans* 1326 and *S. lividans* 1674 where it replicates autonomously with about 50 copies per cell (Hopwood *et al.*, 1985). The activity of two easily and reliably assayed enzymes of the arginine pathway, ornithine acetyltransferase and ornithine carbamoyltransferase (OTC) was measured at different times in the transformants grown in minimal medium (MM) and MM supplemented with 25 mM arginine. The ornithine acetyltransferase activity in the wild-type strain (Fig. 1A) reached a maximum of 2.2 mU mg⁻¹ protein at 36 h. The activity was about 50% lower in cultures supplemented with 25 mM arginine. When *S. lividans* 1326 was transformed with pIJ699 without insert (as control), the ornithine acetyltransferase activity decreased by about 50% in relation to *S. lividans* 1326 in both the presence and the absence of arginine (compare Fig. 1A and B), probably owing to the effect of thiostrepton (10 µg ml⁻¹) used in the cultures as a selective agent to maintain the plasmids. Interestingly, cells of *S. lividans* 1326 [pULML45] (containing the 480 bp ARG box DNA

fragment) showed a higher ornithine acetyltransferase activity (5 mU mg⁻¹ protein at 36 h; Fig. 1B) in spite of the presence of thiostrepton in the culture and were not regulated by the addition of 25 mM arginine for the first 36 h of growth.

The pattern of OTC activity in *S. lividans* 1326 and *S. lividans* [pULML45] cultures is shown in Fig. 1C and D. Arginine (25 mM) repressed the OTC activity by 83% in the wild-type strain at 24 or 36 h. Transformants with plasmid pIJ699 (as control) resulted in a clear decrease in OTC activity, as occurs with the ornithine acetyltransferase, but strains carrying pULML45 showed a 2.2-fold increase in OTC in relation to *S. lividans* 1326 and decreased repression by arginine. Similar results were obtained in *S. lividans* 1674 and *S. lividans* 1674 [pULML45] for both enzymes. These results suggest that amplification of the 480 bp *Sau3A* fragment containing the putative ARG box may titrate out an arginine repressor.

argR, encoding a putative arginine repressor, is linked to *argG* in *S. clavuligerus*

A short incomplete open reading frame, ORF1 in Fig. 2, was found upstream of the *argG* gene in a 6.1 kb *Bgl*III–*Sau3A* DNA fragment from *S. clavuligerus* present in plasmid pULAR10 (Rodríguez-García *et al.*, 1995). To obtain the complete sequence of ORF1, additional clones were isolated from plasmid pULAR10 and an 821-nt DNA fragment upstream of *argG* was sequenced. A complete ORF of 483 nt (Fig. 2B) encoding a protein of 160 amino acids (deduced *M*_r, 17.117 Da, pI 9.5) was found. An inverted repeat nucleotide sequence able to form a stem loop terminator structure in the transcript was located downstream of ORF1 (nt 582–591 and 597–606) with a free energy of -34.5 kcal mol⁻¹; it was followed by a 214 bp intergenic region and by the upstream region of *argG* (ORF2), which contained two putative ARG boxes (boxed in Fig. 2B).

Comparison of the protein encoded by ORF1 showed a clear homology (Fig. 3) with arginine-regulatory proteins of *E. coli* (27.7% identical amino acids and 30.0% additional functionally conserved residues), *S. typhimurium* (28.8% identical amino acids), *H. influenzae* (30.6% identical amino acids) and *B. subtilis* (29.9% identical amino acids). The similarity was particularly high in amino acids 106–110 and 124–134 (motifs d and e in Fig. 3). The eight-amino-acid e motif GTIAGDDTL/I is well conserved in all known arginine repressors. As occurs with the *E. coli* and *B. subtilis* arginine repressors, the N-terminal domain of the encoded protein (amino acids 1–71) has a pI of 11.58 while the C-terminal domain (amino acids 72–160) shows a pI of 4.85. Therefore, the gene corresponding to ORF1 was named *argR*. No ARG box was found upstream of *argR* in the *S. clavuligerus* DNA

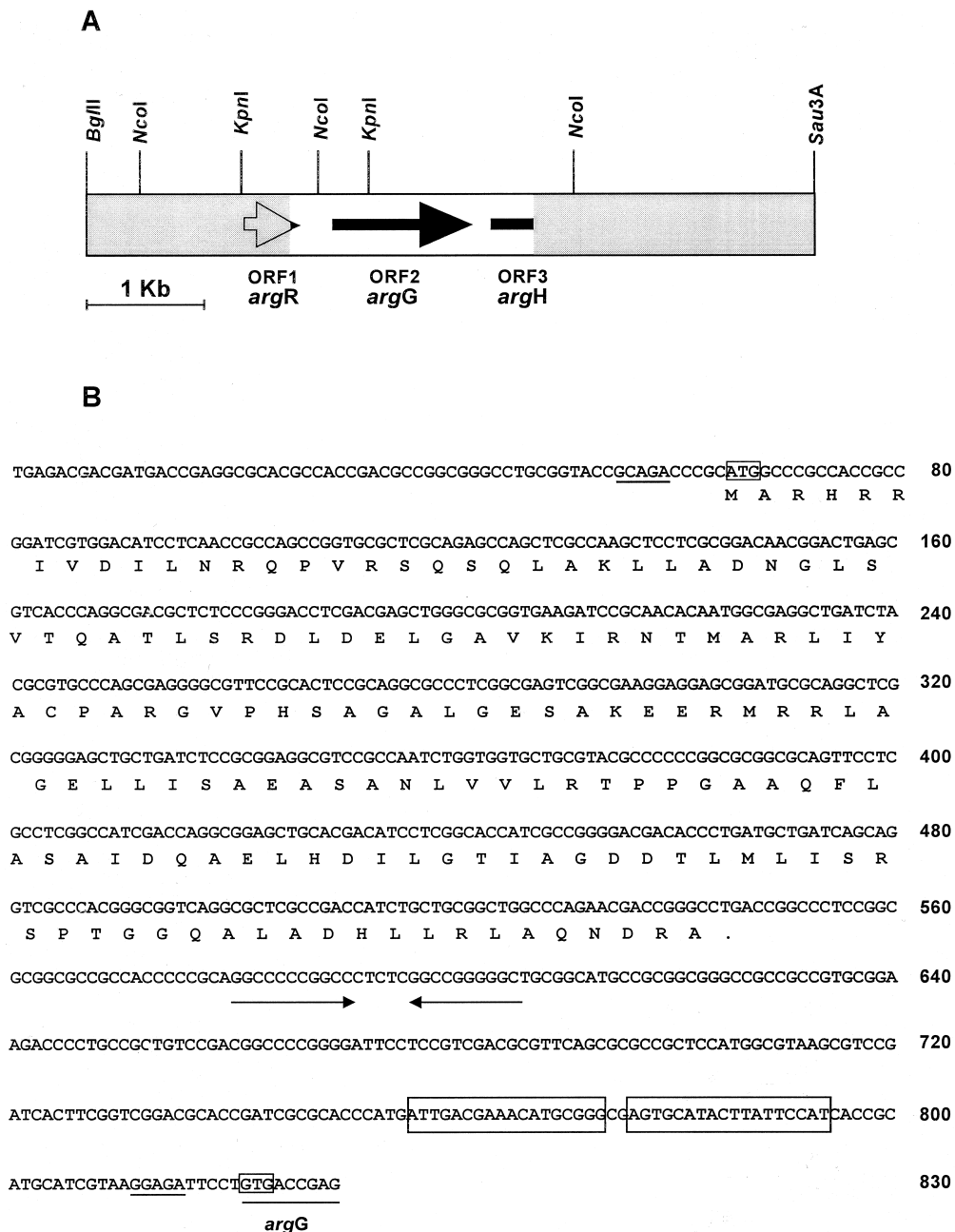


Fig. 2. Restriction map and nucleotide sequence of the *argR* gene of *S. clavuligerus*.

A. Restriction map of a *Bgl*II–*Sau*3A 6.1 kb DNA fragment containing *argR*, *argG* and *argH* genes (arrows). The unshaded area was sequenced previously (Rodríguez-García *et al.*, 1995).

B. Nucleotide sequence and deduced amino acid sequence of a 830 bp fragment containing *argR*. The convergent arrows indicate an inverted repeat (putative transcriptional terminator) motif. Two sequences boxed upstream of the initiation of *argG* correspond to ARG boxes. The translation initiation codons are boxed. Putative ribosome-binding sites are underlined.

fragment sequenced, in contrast to the situation in *E. coli* where *argR* is preceded by an ARG box.

Effect of argR amplification on arginine anabolic and catabolic enzymes

To confirm whether the *argR* gene product has an effect

on arginine biosynthesis enzymes, *S. clavuligerus* was transformed with the multicopy plasmid pULAR12 (containing *argR* in the pIJ101 replicon) and tested for the level of a biosynthetic enzyme, OTC, and a catabolic enzyme, ornithine aminotransferase (OAT), in control (transformed with pULVK99 vector without insert) and pULAR12 transformant strains.

MA---RHRRIV---DILNRQPVRSQSOLAKLLADNGLS-VTQATLSRDIDELGAVKIRN	52	
MNRGQRHIKIR---EIITSNEIETQDELVDMLKQDGYK-VTQATVSRDIKELHLVNVPT	55	
MRSSAKQEELVKAFAKALLKEEFSSQGEIVAAIQEQGFNINQSKVSRMLTKFGAVRTRN	60	
MRSSAKQEELVKAFAKALLKEEFSSQGEIVLALQDQGFENINQSKVSRMLTKFGAVRTRN	60	
MT---ENLTRAFKELLNQEERFGSQSEIVDALKKQGFTEINQSKI SRMLSKFGAVRTRN	55	
TMARLIVAVPAR-GVPHSAGALGESAKEERMRLLAGELLISAEASANLVLRTPPGAQF	111	
NNGSYKYSLPAD---QRFNPLS-----KLKRALMDAFVKIDSASHMIVLKTMPGNAQA	105	
AKMEMVYCLPAELGVPTSSPLK-----NLVLDIDYNDVAVVVVHTSPGAAQL	102	
AKMEMVYCLPAELGVPTSSPLK-----NLVLDIDYNDVAVVVVHTSPGAAQL	107	
TKMEMVYCLPENELSPVNTSSPLK-----NLVLDVDHNAMLIVIKTTPGAAQL	102	
LASAIQAEIHD-ILGTIAGDDTLMILISRSPTGGQALADHLLRLAQNDR	160	<i>S. clavuligerus</i>
IGALMDNLDWDE--MMGTICGDDTILICRTPDETEGVKNRLEL-----L	154	<i>B. subtilis</i> (29.9%)
IARLLDSLGAEGILGTIAGDDTIFFTPANGFTVKDLYEALLELFDQE-L	149	<i>E. coli</i> (27.7%)
IARLLDSLGAEGILGTIAGDDTIFFTPASGFSVRDLYEALLELFEQE-L	154	<i>S. typhimurium</i> (28.8%)
IARLLDSIGKSEGLGTIAGDDTIFFVPTNNKPIDELLQNLQRFENT-L	149	<i>H. influenzae</i> (30.6%)

The biosynthetic OTC activity clearly decreased consistently in a multicopy *argR* transformant *S. clavuligerus* [pULAR12] in agreement with the expected higher intracellular concentration of the ArgR repressor, whereas the catabolic OAT activity increased in the transformants at different times during the culture (Fig. 4A and B). These results suggest that arginine biosynthesis responds negatively to increased copies of *argR*, whereas ornithine deamination (a key step in arginine catabolism) is stimulated.

Interaction of the 480 bp DNA fragment containing the ARG box with AhrC

Since pure ArgR from *S. clavuligerus* is not yet available, we tested whether pure AhrC protein from *B. subtilis* (provided by S. Baumberg) was able to bind to an *S. clavuligerus* ARG box. A *KpnI*-*HindIII* DNA fragment of about 900 bp, which contains the upstream region and the initial part of the *argC* gene, was isolated from plasmid pULML32. The *KpnI*-*HindIII* fragment was digested with *Sau3A*, giving three smaller fragments of about 250, 480 and 150 bp. These fragments were end labelled with [γ - 32 P]-ATP, and the binding to protein AhrC was tested by gel mobility shift. A concentration of 260 nM AhrC produced a clear shift of the 480 bp DNA fragment (containing the putative ARG box) but not of the other two fragments obtained by digestion with *Sau3A* (Fig. 5A, lane 4). AhrC concentrations between 2.6 and 26 nM did not affect the mobility of the bands (lanes 1-3). The purified 480 bp DNA fragment showed a similar behaviour (Fig. 5B). The specificity of the mobility shift was additionally confirmed by using as negative control a 430 bp DNA fragment carrying the *amy* promoter of *S. griseus* and as positive control a 109 bp *HindIII*-*EcoRI* fragment containing the ARG box of *B. subtilis* (not shown).

The AhrC-protected region contains two ARG boxes in tandem

The binding site(s) of AhrC to the upstream region of *argC*

Fig. 3. Comparison of ArgR proteins of different organisms. Identical amino acids are shaded (L and I residues have been considered functionally identical). Asterisks indicate conserved amino acids. Conserved motifs a to f (see text) are overlined. The percentage of identical amino acids to *S. clavuligerus* ArgR is included in parenthesis.

was established by DNase I footprinting experiments. Two types of footprinting analysis were made using 32 P-labelled DNA and fluorescent-labelled DNA respectively. Figure 6 shows the footprinting of AhrC using the 600 bp *KpnI*-*XbaI* and *SacI*-*HindIII* fragments containing the upstream region of *argC*. A protected region of about 30 nt was observed both in the coding strand (Fig. 6A) and in the non-coding strand (Fig. 6B). The addition of arginine (1 mM) to the reaction mixture did not significantly change the footprinting result (Fig. 6A, lane 3, and Fig. 6B, lanes 1 and 2). To establish more precisely the repressor-operator interaction, a footprinting procedure using fluorescent-labelled DNA was used. Results of footprinting using fluorescent-labelled primers are shown in Fig. 7. A 38-nt region [ATTGCATAAAAGTGCAGT]GA[TTTGATAGTCATGCCTT] (Fig. 7A) was protected in the coding strand. Similarly, a 36-nt sequence [CATGACTATACA-AATCACTGCACTTTTATGCAATGA] (Fig. 7B) was protected by AhrC in the non-coding strand.

Comparison of the protected DNA region with the ARG box of *E. coli* showed the presence of two ARG boxes (18 nt each) repeated in tandem in the protected sequence

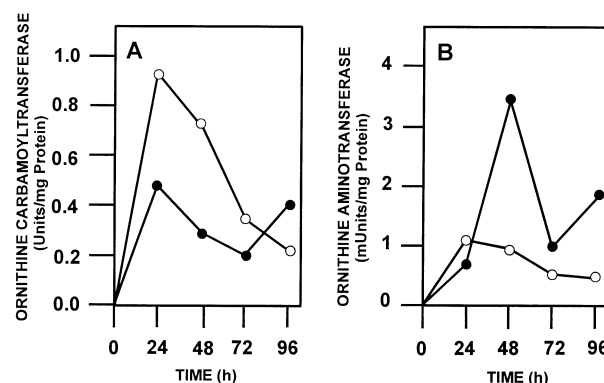


Fig. 4. Effect of *argR* amplification on enzyme activities by *S. clavuligerus* transformants. A. Ornithine carbamoyltransferase activity. B. Ornithine aminotransferase. *S. clavuligerus* [pULVK99] (○); *S. clavuligerus* [pULAR12] (●).

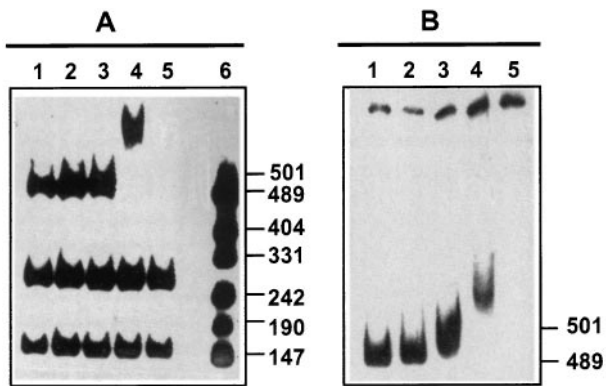


Fig. 5. Mobility shift of DNA fragments containing an ARG box in the presence of AhrC protein. A. Retardation of DNA fragments obtained by *Sau*3A digestion of the *Kpn*I–*Hind*III DNA fragment carrying the ARG box. B. Retardation of the isolated 480bp *Sau*3A fragment. Concentrations of AhrC were as follows: lane 1, no addition; lane 2, 13 nM; lane 3, 26 nM; lane 4, 260 nM; lane 5, 1300 nM; lane 6, size markers (pUC19/*Hpa*II) in bp are indicated on the right.

(Fig. 7). As observed in Fig. 7A and B, the protected region in the coding and non-coding strand is displaced 2 nt in the 5' end and 4 nt in the 3' end of the ARG box (Fig. 7C). In the centre of the protected sequence, nucleotides CAG (coding strand) and GCAC (non-coding strand) appeared to be incompletely protected. The presence of partially protected nucleotides was also observed in Fig. 6A and B.

ARG boxes in *Streptomyces* genes

The consensus sequence of all known ARG boxes and its inverse in *E. coli* is:



(Charlier *et al.*, 1992). Normally there are two ARG boxes separated by 3 bp, and only in the *E. coli argR* ARG boxes is the separation of 2 bp. Using this information, an ARGBOX.MAT matrix was constructed and used to search the 819 *Streptomyces* genes in the GenBank database (DNASTar), looking for a pattern of two matrices separated by 1–4 nucleotides. The search, using a detection threshold average of 69%, detected the presence of seven promoter regions containing ARG boxes in the *Streptomyces* sequences. All of them contain two consecutive ARG boxes and are shown in both the coding and the non-coding strand in Fig. 8. Most of these sequences correspond to genes involved in arginine biosynthesis, such as *argC* and *argG* of *S. coelicolor*, *argG* of *S. lavendulae* and *argC* and *argG* of *S. clavuligerus*. Other promoter sequences detected are not directly related to arginine biosynthesis, such as the promoter of ORF4 in the clavulanic acid

gene cluster of *S. clavuligerus*, encoding an ornithine acetyltransferase-like enzyme (Hodgson *et al.*, 1995) or the SEP8 promoter of an unknown gene of *S. lividans* cloned by Forsman and Jaurin (1987).

The percentage of every base was calculated in all positions of the ARG boxes shown in Fig. 8. A specific nucleotide is considered to be consensus when it appears in a defined position more than 65% of the time; two alternative nucleotides (as in position 8) when each is found between 30% and 50% of the time; finally, in some positions (such as positions 1 or 18), one nucleotide is present about 40% of the time and all the others are proportionally represented. Using these rules, which were also used to define the consensus *E. coli* ARG box (when using *E. coli* ARG box sequences), the following *Streptomyces* consensus ARG box was obtained:

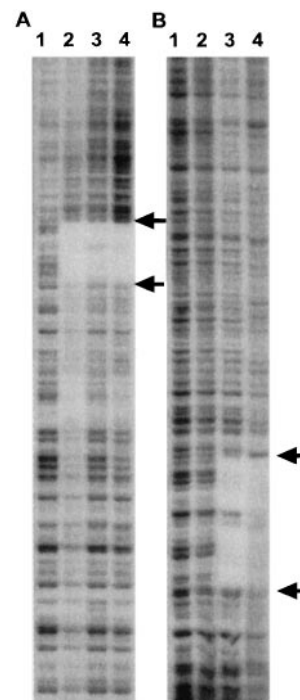


Fig. 6. Footprinting using ³²P-labelled DNA. A. Coding strand. Reactions were incubated as follows: lane 1, 20 s in the presence of arginine; lane 2, 30 s in the presence of AhrC; lane 3, 30 s in the presence of AhrC and arginine; lane 4, 20 s in the presence of arginine and AhrC. B. Non-coding strand. Incubations were as follows: lane 1, 30 s in the presence of arginine and bovine serum albumin (100 µg ml⁻¹); lane 2, 30 s in the presence of arginine; lane 3, 30 s in the presence of AhrC; lane 4, 30 s in the presence of AhrC and arginine. AhrC concentrations were 1 µM for the coding strand and 0.5 µM for the non-coding strand. Arginine concentration, when added, was 1 mM.

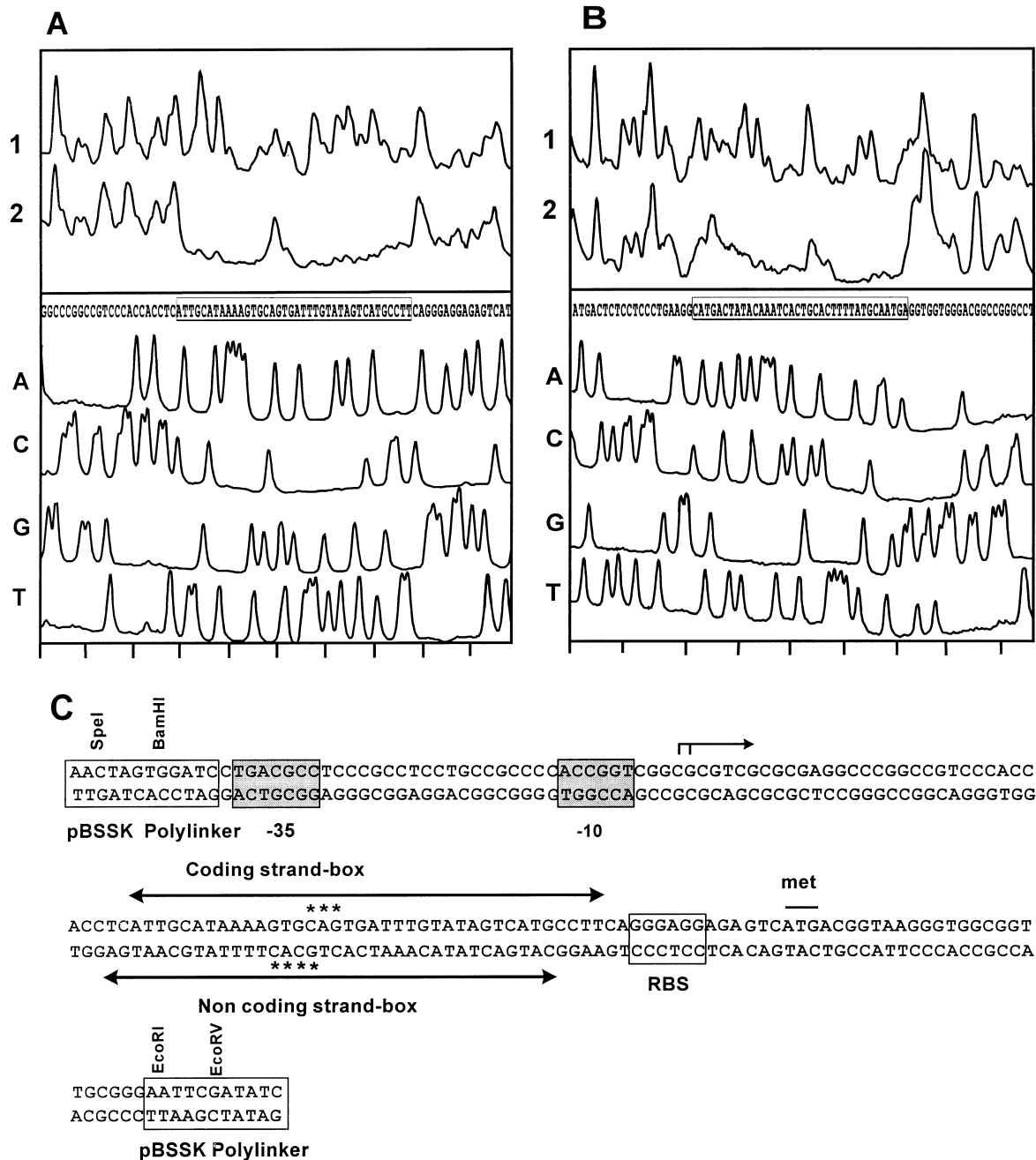


Fig. 7. Footprinting of the ARG box upstream of *argC* of *S. clavuligerus* with AhrC protein (0.5 μ M) using the fluorescent-labelling procedure. A. Coding strand. B. Non-coding strand. The pattern sequence of (1) control (no AhrC added); and (2) the sequence in the presence of AhrC (0.5 μ M) is shown in the upper panels. The lower panels show the nucleotide sequence. C. DNA fragment used for the footprinting containing 148 nt of the upstream region of *argC* in pBSSK(+). The protected sequences in the coding and non-coding strands are shown by arrows. The unprotected nucleotides are indicated by asterisks.

As observed, the consensus is more degenerate at the ends and in the centre of the ARG box. Two stretches of nucleotides (nt 3–7 and 12–16) are well conserved. When these two regions are compared with the homologous sequences in the consensus *E. coli* ARG box, at

least eight positions (nt 3–4, 6–7, 12–13 and 15–16) are found perfectly conserved.

	3	4	5	6	7	12	13	14	15	16
<i>E. coli</i> consensus	T	G	A	A	T	A	T	T	C	A
<i>Streptomyces</i> consensus	T	G	C	A	T	A	T	G	C	A

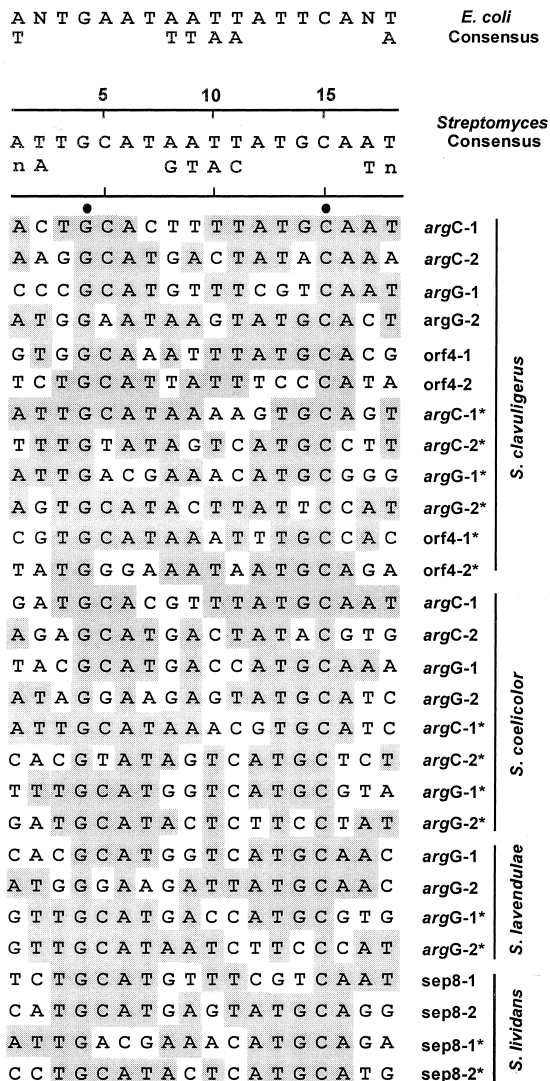


Fig. 8. ARG boxes found in *Streptomyces* sequences using the ARGBOX.MAT matrix. Conserved amino acids are shadowed. The *E. coli* consensus and *Streptomyces* consensus ARG boxes are indicated in the upper part of the figure. The nucleotides conserved in all (100%) ARG boxes are indicated by a dot (●).

Nucleotides at positions 4 and 15 are present in all (100%) the *Streptomyces* ARG boxes found in databases (Fig. 8) and in 94% of *E. coli* ARG boxes (the substitutions are always of one purine by another or one pyrimidine by another). This indicates that positions 4 and 15 (indicated by dots in Fig. 8) are especially important for ArgR binding to ARG boxes in *Streptomyces*, as was demonstrated experimentally by Charlier *et al.* (1992) for the same residues in *E. coli* ARG boxes.

Discussion

In *E. coli*, *argR*, encoding the arginine repressor, is located at -71 of the map and is unlinked to other arginine genes.

In *B. subtilis*, several genes of the arginine and pyrimidine biosynthesis pathway are linked in two clusters, *argCAE-BDcarABargF* and *argGH* (Mountain *et al.*, 1984; North *et al.*, 1989), while *ahrC*, encoding the repressor, is located in a separated 6 kb *EcoRI* DNA fragment. As shown in this article, in *Streptomyces clavuligerus*, *argR* is linked to *argG*. It forms part of a cluster of arginine biosynthesis genes containing (at least) *argRGH* (Rodríguez-García *et al.*, 1995). In *S. coelicolor*, the unstable *argG* is not preceded by *argR* (Ishihara *et al.*, 1985).

The deduced ArgR protein in *S. clavuligerus* shows a monomer molecular weight of 17 117, similar to those of the *E. coli* ArgR and *B. subtilis* AhrC (monomer sizes are 16 995 and 16 833 respectively), which form a hexameric structure with molecular mass of 98 and 96 kDa respectively. Purification of *S. clavuligerus* ArgR is in progress to establish whether it behaves as a hexamer.

In *E. coli* repressor, the C-terminal domain of ArgR (amino acids 80 to the end, pI 3.70) has been crystallized and shown to contain the arginine-binding domain that is responsible for oligomerization (Van Duyne *et al.*, 1996). The *S. clavuligerus* repressor shows three highly conserved motifs (d, e and f in Fig. 2) in this C-terminal moiety. The amino-terminal moiety (amino acids 1–71) of the *S. clavuligerus* ArgR protein shows a pI of 11.58 and corresponds to the DNA-binding motif of the *E. coli* ArgR repressor, whereas the C-terminal region shows a pI of 4.85.

Overexpression of *argR* in *S. clavuligerus* resulted in a clear repressive effect on the activity of the biosynthetic OTC, but it produced a stimulation of the catabolic OAT. These results suggest that, in *Streptomyces* species, ArgR represses the arginine biosynthesis genes and activates (at least some) arginine catabolic genes, as occurs also with AhrC in *B. subtilis* (Klingel *et al.*, 1995).

In *S. clavuligerus*, *argR* is not preceded by an ARG box, in contrast to the situation in *E. coli*, suggesting that expression of *argR* may not be autoregulated. Amplification of a standard ARG box in a multicopy plasmid in *S. lividans* resulted in a derepression of the biosynthetic activities of 2- to 2.5-fold, suggesting that the repressor is titrated out. A similar effect has been reported by Soutar and Baumberg (1996) in *S. coelicolor*. In *E. coli*, 300 copies of the arginine repressor are known to exist per cell (increasing to about 1000 under arginine starvation conditions). If a similar number of ArgR molecules occurs in *S. clavuligerus*, a significant part may be titrated out by an ARG box in multicopy pIJ101- derived plasmids that are present at the rate of 40–300 copies per cell (Hopwood *et al.*, 1985).

The functionality of the ARG boxes of *S. clavuligerus* has been proved by band mobility shift using the purified AhrC repressor (no pure homologous ArgR is available). AhrC from *B. subtilis* has also been found to interact with

Table 1. Plasmids used in this work.

Plasmid	Size	Characteristics	Reference
pULAR12	9.3 kb	pJVK99 containing the <i>argR</i> gene in a 1.5 kb <i>NcoI</i> fragment	This work
pULAR10	9.0 kb	pBSSK(+) containing a 6.1 kb DNA fragment carrying <i>argG</i> and <i>argR</i>	Rodríguez-García <i>et al.</i> (1995)
pULML3255	3.5 kb	Fragment <i>Sau3A</i> of 480 pb inserted in the <i>Bam</i> HI site of pBSSK(+)	This work
pULML45	10.0 kb	pIJ699 carrying in its <i>Bgl</i> II site a 480-pb <i>Sau3A</i> fragment containing <i>S. clavuligerus</i> ARG box	This work
pULMA	3.1 kb	pBSSK(+) containing a 148-pb <i>Bam</i> HI– <i>Eco</i> RI DNA fragment obtained by PCR from pULAR3255	This work
pIJ699	9.6 kb	Bifunctional <i>E. coli</i> – <i>Streptomyces</i> positive selection vector. <i>tsr aph, vph</i>	Kieser and Melton (1988)
pULVK99	7.8 kb	Bifunctional <i>E. coli</i> – <i>Streptomyces</i> positive selection vector. <i>tsr kan</i>	V. Kumar and J.F. Martín (unpubl. results)

the promoter region of *argC* of the thermophilic strain *B. stearothermophilus* (Savchenko *et al.*, 1996). The excellent binding of AhrC to the *S. clavuligerus* ARG boxes and the specificity shown in comparison with positive and negative controls clearly indicates that the ARG boxes located upstream of the *argC* gene of *S. clavuligerus* are indeed recognized by the arginine repressor. Further support for this conclusion has been provided by the footprinting studies using two different protocols. In addition to the standard footprinting, as shown by DNase I digestion, we have developed a novel procedure (A. Becker, personal communication) using fluorescent-labelled DNA. The protected region is a 38-nt stretch that contains two imperfect palindromic sequences corresponding to the ARG boxes of *E. coli*. Each ARG box contains 18 nt separated by 2 nt (as opposed to 3 nt in *E. coli*). Inside the ARG boxes, a CAG triplet in the coding strand and a GCAC in the non-coding strand are less preserved as observed clearly in both protection experiments. Similar results were also reported in the ARG boxes of *E. coli* and *B. subtilis* (Charlier *et al.*, 1992; Czaplewski *et al.*, 1992; Tian *et al.*, 1992). This observation suggests that the arginine repressor binds to both ends of the ARG box leaving the pocket accessible to the DNase I in the centre.

Experimental procedures

Strains and culture conditions

DNA of *Streptomyces clavuligerus* NRRL 3585, producer of cephamycin C and clavulanic acid, was used to clone the arginine-regulatory region. *Streptomyces lividans* J11326, wild-type strain, and *Streptomyces lividans* 1674, an *argC* auxotroph (Ludovice *et al.*, 1992), were used as hosts for transformation and complementation studies. To measure enzyme activities, *S. lividans* strains were grown in MM (Hopwood *et al.*, 1985). An aliquot of 2.5 ml of a 48-h culture was used as seed to inoculate flasks with 50 ml of MM, and the culture was grown at

28°C and 250 r.p.m. Cultures of *S. lividans* 1674 and transformants from this strain were supplemented with 0.5 mM arginine. Alternatively, to measure the effect of *argR* on the arginine biosynthetic enzymes of *S. clavuligerus* transformants, seed cultures were grown for 36 h in MI medium containing 20 g l⁻¹ glycerol, 10 g l⁻¹ bacto-peptone, 10 g l⁻¹ malt extract, pH 7.0, supplemented with kanamycin (5 µg ml⁻¹) when required. Flasks containing 100 ml of GSPG medium (Romero *et al.*, 1984) were inoculated with 5% v/v seed culture and incubated at 28°C in an orbital incubator at 220 r.p.m.

Plasmids and transformation of *S. clavuligerus* and *S. lividans*

Protoplasts of *S. clavuligerus* and *S. lividans* were transformed using the procedures of García-Domínguez *et al.* (1987) and Hopwood *et al.* (1985). All plasmids used in this work are listed in Table 1.

Cell extracts and enzyme activities

S. clavuligerus or *S. lividans* cultures (50 ml) were centrifuged at 10 000 × g for 10 min, washed twice with 0.9% NaCl and suspended in 2.5 ml of 0.1 M phosphate buffer, pH 7.0. The cells, kept at 4°C, were disrupted by sonication using 30-s pulses with 2-min intervals (with refrigeration in an ice bath) for a total time of 120 s in a Branson B12 Sonifier. The cell-free extract was centrifuged at 17 000 r.p.m. for 30 min at 4°C, and enzyme activities were measured in the supernatant.

Ornithine acetyltransferase. This was measured in undialysed cell-free extracts, as indicated by Vogel and Bonner (1956) for *N*-acetylornithinase. A unit is defined as the enzyme activity able to form 1 µmol of ornithine per minute.

Ornithine carbamoyltransferase (OTC). The assay of OTC (forward reaction) was performed as indicated by Fuente *et al.* (1996). One unit is defined as the activity that catalyses the formation of 1 µmol of citrulline per minute.

Ornithine aminotransferase (OAT). The OAT activity was assayed according to Jenkins and Tsai (1970). One unit is defined as the activity that forms 1 μmol of Δ^1 -pyrroline-5-carboxylic acid per minute (measured by the absorbance at 440 nm of the o-aminobenzaldehyde derivative formed).

Gel retardation assays

Protein–DNA complexes were examined by gel retardation assays. A 1.0 kb *HindIII*–*KpnI* DNA fragment containing the ARG box of the *argC* gene of *S. clavuligerus* was digested with *Sau3A* and the fragments used for protein–DNA interaction studies. Alternatively, the 480 bp *Sau3A* DNA fragment containing the ARG box was isolated from agarose gels using the method of Langridge *et al.* (1980). DNA fragments were end labelled with [γ - ^{32}P]-dATP using T4 polynucleotide kinase in a reaction mixture containing in FB buffer (10 mM Tris-HCl, pH 7.4, 5 mM MgCl₂, 2.5 mM CaCl₂, 250 mM KCl and 0.5 mM dithiothreitol), sonicated salmon sperm DNA (50 $\mu\text{g ml}^{-1}$) and 1 mM L-arginine in a final 18- μl volume. AhrC (0–260 nM) was added and incubated for 20 min at 37°C; then 2 μl of glycerol (50%) was added to the mixture and incubated for an additional 10 min. Protein–DNA complexes were resolved by PAGE (6% polyacrylamide).

Radioactive footprinting

DNase I footprinting with AhrC protein was carried out according to Galas and Schmitz (1978). *SacI*–*HindIII* and *KpnI*–*XbaI* DNA fragments (both of 600 bp), carrying the ARG box of *argC*, were obtained from plasmid pULML3255 (the restriction sites belong to the polylinker). DNA from each fragment (250 ng) was end labelled with [α - ^{32}P]-dCTP (3000 Ci mmol⁻¹, 10 mCi ml⁻¹) in the presence of dGTP, dTTP, dATP (7.5 μM each), 1.2 units of DNA polymerase (Klenow) and commercial buffer PolK (10 \times), pH 7.5 (Boehringer), in a total volume of 20 μl . After 10 min at 37°C, the labelled bands were purified in a Magic Column (Promega). Purified labelled fragments (10 ng) were mixed with AhrC protein (0.5 or 1 μM) and incubated for 20 min at 37°C in FB buffer containing 50 $\mu\text{g ml}^{-1}$ salmon sperm DNA and 1 mM arginine. DNase I (Boehringer) was then added (final concentration 0.0062 units per reaction), and the digestion was allowed for 15–60 s and then stopped by the addition of 40 μl of stop buffer (5 M ammonium acetate, 125 μM EDTA, 125 $\mu\text{g ml}^{-1}$ tRNA, pH 8.0). DNA was denatured at 95°C for 2 min and applied (4 μl) to a polyacrylamide sequencing gel.

Protection experiments using fluorescent-labelled DNA

The protection was performed according to A. Becker (personal communication). A 600 bp *BamHI*–*EcoRI* DNA fragment was amplified by polymerase chain reaction (PCR) using as template, pULML3255, and probes: P(+): [CCGGATCCTG-ACGCCTCCCGCCTCCTGC] and P(–): [ACGAATTCCCG-CAACCGCCACCCTTACCG] (which contain a *BamHI* and an *EcoRI* site respectively) in a DNA thermal cycler (Perkin-Elmer Cetus). Conditions for PCR were as follows: 1.5 mM MgCl₂, 42.6 μM dGTP and dCTP, 20 μM dATP and dTTP, 0.2 μM probes, 0.1 ng DNA template, 2 units *Taq* DNA polymerase and Perkin-Elmer PCR buffer to a final volume of

100 μl . The reaction mixture was denatured at 97°C for 35 s followed by five PCR cycles of 97°C for 15 s, 70°C for 30 s, 76°C for 5 s and 27 cycles of 97°C for 15 s, 62°C for 20 s and 72°C for 1 s with increasing times of 1 s per cycle and a final cycle of 76°C for 10 min. The 600 bp *BamHI*–*EcoRI* fragment obtained was inserted in the polylinker of pBSSK(+) to give plasmid pULMA.

Fluorescent-labelled DNA was obtained by PCR using pULMA as template, fluorescent-labelled universal probe (24 nt) (Pharmacia) and non-labelled reverse probe (17 nt) (Genosys); the complementary strand was labelled using labelled 17 nt reverse probe (Pharmacia) and non-labelled 17 nt universal probe (Genosys). The reaction mixture for PCR contained 300 μM dGTP and dCTP, 200 μM dATP and dTTP, 0.4 μM probes, 133 μM DNA template, 10 units of *Taq* DNA polymerase and buffer 12 from Opti-Prime PCR Optimization Kit (Stratagene) to a final volume of 300 μl . A PCR cycle of 94°C for 3 min and 55°C for 1 min was followed by 35 cycles of 72°C for 40 s, 94°C for 60 s, 55°C for 30 s and finally one cycle of 72°C for 10 min. The PCR products were extracted from the 1.6% agarose gel using Qiaex II (Qiagen) and suspended in FB buffer at 100 $\mu\text{g ml}^{-1}$ concentration.

For AhrC–DNA binding studies, 100 ng of fluorescent-labelled DNA was suspended in FB buffer containing 0.5 mM arginine, and AhrC was added at final concentrations of 0.125, 0.25, 0.5 and 1 μM . After incubation at 37°C for 30 min, 0.01 units of DNase I (grade I, Boehringer) was added and allowed to react for 1 min at 37°C. The reaction was stopped with 180 μl of TE buffer containing 40 mM EDTA, pH 8.0. The DNA was purified by standard methods and sequenced in an ALF-DNA Sequencer (Pharmacia).

Sequence analysis

Comparison of proteins was made using the Lipman–Pearson algorithm and the CLUSTALV programs from DNASTar. ARG box sequences were searched with the PATTERNS and GENEMAN programs from DNASTar. The nucleotide sequence of *argR* has been deposited in the EMBL database with the accession number Y11134.

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