Characterization and Expression of the Arginine Biosynthesis Gene Cluster of Strepotmyces clavuligerus

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Abstract

A cluster of genes argCJBDRGH containing most of the arginine biosynthesis genes has been found in Strepotmyces clavuligerus after sequencing a 8.3 kb DNA region containing overlapping sequences of two DNA fragments known to contain arginine biosynthesis genes. Subcloning, complementation of E. coli arginine auxotrophic strains and enzymatic assays confirmed the identity of each gene. S1 nuclease mapping studies and Northern hybridization analysis revealed the formation of two large transcripts corresponding to argCJBDR and argGH. The amount of each of these mRNAs is 10 to 44 times higher in a S. clavuligerus argR-disrupted mutant than in the wild type confirming the existence of an ArgR-mediated control of arginine biosynthesis gene expression. A low level constitutive monocistronic transcript of argR was observed in S. clavuligerus cells. Most of the argGH transcript initiating at an adenine 29 nt upstream of the argG initiation codon appears to stop at a termination stem and loop structure present downstream of the argG gene.

Introduction

Arginine biosynthesis genes (arg) and genes for carbamoyl phosphate (required for citrulline) biosynthesis (car) are usually scattered in the genome of most Gram negative bacteria. In E. coli there is an argECBH cluster, in which the argE and argCBH are transcribed divergently (Crabeel et al., 1979). The carAB genes are also clustered, while other genes (argA, argB, argD, argL, argF, argG) map at different positions in the E. coli chromosome. The argECBH cluster is also found in Salmonella typhimurium (Sanderson, 1970) while in Proteus mirabilis and Serratia marcescens includes additionally argG upstream of argH (Prozesky 1968, Matsumoto et al., 1975). In Neisseria gonorrhoeae and Pseudomonas aeruginosa all the arg genes are scattered (Picard and Dillon, 1989; Haas et al., 1977).

In Gram positive bacteria clustering of arginine genes appears to be common. In Lactobacillus plantarum a carAargCJBDF cluster has been described (Bringel et al., 1997) with divergent transcription for the car and the arg genes. The same organization for arginine genes (argCJBDF) was described in Corynebacterium glutamicum (Sakanyan et al., 1996); in Mycobacterium tuberculosis and Mycobacterium leprae, DNA sequences for a argCJBDFRGH cluster have been deposited in EMBL database (Accession numbers Z85982 and L78811, respectively). Arginine biosynthesis genes in Bacillus subtilis are organized in two operons, one is argCJBDNArcAB-carF for early steps in the arginine pathway, including those involved in carbamoyl phosphate biosynthesis (Mountain et al., 1984; O’Reilly and Devine, 1994), and a second operon argGH encodes enzymes for the late steps of the pathway (Piggot and Hoch, 1985). An argE gene has been located by sequencing the B. subtilis genome (Kunst et al., 1997) but its relation with the cyclic pathway of arginine biosynthesis in this bacterium (Figure 1) remains to be elucidated; the regulatory gene ahrC encoding the arginine biosynthesis repressor (North et al., 1989) is located in a separate region. This organization in two separate operons may occur also in other Gram positive bacteria and could explain the lack of information on the location of argG, argH and argR in Lactobacillus and Corynebacteria.

Arginine defective mutants of Strepotmyces coelicolor have been mapped as scattered at four different locations in the chromosome (Redenbach et al., 1996). By complementation of E. coli arginine defective mutants a cluster argCJB has been located in S. coelicolor (Hindle et al., 1994) whereas the argGH gene is located in an unstable region at the end of the lineal chromosome of this actinomycete (Redenbach et al., 1996). Fragments of S. clavuligerus genome were also able to complement E. coli arginine mutants and the subsequent sequencing of those fragments revealed the presence of two subclusters of arginine genes in this actinomycete: argCJ (Ludovice et al., 1992) and argRGH (Rodriguez-García et al., 1995; 1997).

An important question from the regulation point of view is if the two clusters organization occurs in all actinomycetes and how expression of these clusters is controlled by the ArgR repressor. This information is particularly important in S. clavuligerus, since this actinomycete uses arginine as precursor for clavulanic acid biosynthesis (Valentine et al., 1993). Knowledge of the arginine gene cluster organization and their regulatory mechanisms (Rodriguez-García et al., 1997) is important to understand how the flow of arginine affects clavulanic acid production.

In this paper we provide evidence showing that most of the arginine genes in S. clavuligerus are present in a single argCJBDRGH organization, which includes genes for the early and late steps of the arginine pathway as well as the regulatory gene argR. We have also shown that carAorf4, in the clavulanic acid cluster, encodes a second functional ornithine N-acetyltransferase similar to ArgJ.
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Results

Location of the Arginine Gene Cluster of S. clavuligerus

The argC gene was initially located by sequencing a 6.1 kb Sau3AI DNA fragment of S. clavuligerus present in plasmid pULML31, which complements the argC deficient mutant E. coli XC33 (Ludovice et al., 1992).

Independently, a 18 kb Sau3AI DNA fragment of S. clavuligerus was found to complement E. coli argG deficient mutants (Rodríguez-Garcia et al., 1995). Further studies showed the presence in this DNA fragment of an argRGH cluster (Rodríguez-Garcia et al., 1997).

In order to establish the definitive organization of the arginine biosynthesis genes, a 0.5 kb SacI-SphI DNA fragment (Figure 2A) located downstream of argC in pULML31 was mapped and partially sequenced. In parallel, a 2.1 kb SacI-BamHI DNA fragment from pULAR1, upstream of argR (Figure 2A), was also sequenced. The nucleotide sequence of both fragments was found to overlap, indicating that argC and argR (previously though to be in two different clusters) are closely located in the S. clavuligerus genome. Therefore, a DNA region, covering 8.3 kb was sequenced in both orientations.

Analysis of the arg Cluster

The G+C content of the sequenced fragment was 71.3%, with no significant differences of G+C in the intergenic regions. Rare TTA codons for leucine were absent from the coding regions, indicating that these genes are not regulated by the bldA-dependent translation control (Leskiw et al., 1991). Analysis of the nucleotide sequence of the entire fragment using the Geneplot Program of DNAstar revealed the presence of eight ORFs, oriented in the same direction. Comparison of the amino acid sequences deduced from the ORFs with the sequences of arginine metabolic enzymes shows that ORF1 to ORF7, correspond to arginine biosynthesis genes. These genes include, as expected, argC (nt 123-1148) which correspond to ORF1, argR and argG (nt 4600 to 6604) corresponding to ORF5 and ORF6. The proteins encoded by ORF2, ORF3 and ORF4 were homologous with ArgJ, ArgB and ArgD of other organisms while ORF7 encodes argH. ORF8 was truncated and its deduced amino acid sequence does not correspond to any enzyme of the arginine biosynthesis pathway.

Putative ribosome binding sites were found upstream of argJ, argB, argD and argH, but according to the Streptomyces promoter criteria (Strohl, 1992) no clear −10 and −35 boxes were found upstream of the newly described ORFs. Two inverted sequences able to form a stem and loop structure with free energy of −37.2 kcal/mol were found downstream of argG (nt 6628-6640/6650-6662). They might act as a transcription terminator of the argG gene. Similar putative terminator sequences were present downstream of argH (nt 8162-8172/8182-8192 and 8171-8178/8183-8190) giving stem and loop structures with free energies of −20.2 and −12.6 kcal/mol.

argJ Encodes a Functional Ornithine N-Acetyltransferase: Evidence for a Cyclic Arginine Pathway

argJ (ORF2) has 1,152 nt and encodes a protein of 383 amino acids with a deduced Mr of 39,733. The amino acid sequence of this protein showed the highest similarity (74.9% identical residues) with the unpublished sequence of ArgJ of S. coelicolor (Sanger cosmid SCL24) followed by the ornithine acetyltransferases of M. tuberculosis (47.8% identity) and C. glutamicum (40.7% identity).

E. coli contains N-acetylornithinase but not the argJ-encoded ornithine N-acetyltransferase. Bifunctional ornithine N-acetyltransferases (EC 2.3.1.35) with N-acetylornithine (EC 3.5.1.16) activity have been reported in some microorganisms. In order to test whether the argJ gene of S. clavuligerus confers acetylornithinate activity a 2.1 kb SacI-BamHI DNA fragment containing argJ was subcloned in both orientations in pBSKS(+) (giving plasmids pKS21.1 and pKS21.2) and introduced into E. coli XS1D2, an E. coli mutant lacking acetylornithinase activity. Ornithine N-acetyltransferase activity (0.76 units/mg protein) was found in cell-free extracts of E. coli XS1D2 in which the S. clavuligerus argJ was expressed from the lacZ promoter but no activity was found in E. coli XS1D[pKS21.2] in which the gene was subcloned in the opposite orientation. No N-acetylornithinase activity was found in any of the transformants. These results confirm that S. clavuligerus contains a cyclic N-acetyltransferase pathway as reported by Hindle et al. (1994) in S. coelicolor, and not the linear (E. coli type) pathway.

A Second Gene Encoding an N-Acetylornithine Acetyltransferase Occurs in the Clavulanic Acid Cluster

ArgJ shows a 31.1% identity in amino acids over the entire protein sequence with the protein encoded by ORF4 of the clavulanic acid gene cluster (hereafter named ca-ORF4) of S. clavuligerus (Hodgson et al., 1995). The amino acid sequence of the protein encoded by ca-ORF4 contains most of the conserved boxes found in ArgJ proteins. However the similarity of the protein encoded by ca-ORF4 with the ArgJ protein of S. clavuligerus (31.1%) is lower than the similarity between ArgJ proteins of other Gram
positive bacteria. In order to test if the protein encoded by ca-ORF4 shows ornithine N-acetyltransferase activity, a 4.1 kb KpnI-NruI DNA fragment containing ca-ORF4 was subcloned in pBSKS(+) to give plasmid pBS41. Cell-free extracts of the transformant E. coli XSD1[pBS41] showed an ornithine N-acetyltransferase activity of 1.79 units/mg protein indicating that promoter sequences upstream of ca-ORF4 are expressed in E. coli and confirming that the gene encodes an ArgJ-homologous protein with ornithine N-acetyltransferase activity but no acetylornithinase activity. Therefore we confirm that in S. clavuligerus there are at least two genes encoding ornithine N-acetyltransferases (Eascott et al., 1998). The low amino acid identity of the protein encoded by ca-ORF4 with other ArgJ proteins suggest that the role of this gene in S. clavuligerus might not be related to arginine biosynthesis, but perhaps involved in modification of arginine (e.g. acting as arginine N-acetyltransferase) as a way to commit it to clavulanic acid biosynthesis.

The Protein Encoded by argB Complements argB Mutants of E. coli

The argB (ORF3, 909 nt) gene has a G+C content of 68.5%, and encodes a protein of Mr 32,365. The N-acetylglutamate kinase protein (EC 2.7.2.8) has 302 amino acids and a sequence similar to the homologous proteins of C. glutamicum (50.0% amino acid identity) and M. tuberculosis (47.8% identity). A motif conserved in all the ArgB proteins is present upstream of the argG gene (VHGGGPXI...). To confirm the identity of the gene, plasmid pULB, containing the argB gen, was used to complement the argB-deficient strain E. coli XB25. Transformant E. coli [pULB] was able to grow in VB minimal medium while the control strain E. coli XB25[pBSKS+] was not.
Characteristics of argD
ORF4 (1203 nt with a G+C content of 71.3%) encodes a 400 amino acid protein with a calculated Mr of 41,972. The argB stop codon overlaps with the initiation codon of ORF3. A search in the Swiss-Prot database with the FASTA Program revealed that the protein encoded by ORF3 has a high similarity to acetylornithine aminotransferases (ACOAT) and ornithine aminotransferases (OAT) (54.5% identity with the ArgD of C. glutamicum and 47.8% with that of C. glutamicum).

The sequence 214LVLDVQGIGRTGHWFAMAQAEVEADVVTALAKLGGS267 corresponds to the pyridoxal phosphate (PP) binding motif, with K246 as the conserved lysine for covalent PP-binding (Yonaha et al., 1992). Multiple alignment of the ORF4-encoded protein with ten known ArgD proteins showed the presence of domains conserved in ACOAT's and OAT's, but the amino acid similarity of the ORF4-encoded protein is higher to ACOATs of B. subtilis and E. coli than to the corresponding OAT proteins. This observation is consistent with the amination of N-acetylglutamic semialdehyde to N-acetylornithine (which occurs when the intermediate is in the N-acetylated form).

In order to confirm that the cloned gene corresponds to argD, plasmid pULD was used to transform E. coli CGSC 4538, an argD proA double mutant. The ampicillin resistant transformants were able to grow in VB medium supplemented with proline but also in VB medium without supplementation with either proline or arginine. Since the proA mutation precludes the formation of glutamate-γ-semialdehyde required for proline biosynthesis, the complementation of both proA and argD phenotypes by the argD gene of S. clavuligerus suggests an interconversion of intermediates of the proline and arginine pathways.

Characterization of argH
ORF7 (1,422 nt), encoding the argH gene (argininosuccinate lyase) was described to be located downstream of argG by partial sequencing of the gene (Rodríguez-García et al., 1995). It is preceded by a ribosome binding motif AGGAG. The protein ArgH has a calculated Mr of 50,915. The amino acid sequence shows the highest similarity with ArgH of M. tuberculosis (60.4% amino acid identity) followed by the homologous human and rat proteins (43 and 42% identity). The amino acid sequence 232GSSIMPQKN241 found in the protein encoded by ORF7 corresponds to the GSXXMXXKN motif characteristic of all fumarate lyases in which the central methionine residue appears to be involved in the active center of the enzyme (Woods et al., 1988).

The argH gene present in pULAR11 downstream of argG was used to transform E. coli CGSC 5359, an argH auxotroph. E. coli 5359[pULAR11] ampicillin resistant transformants grew in VB minimal medium supplemented with ampicillin, while the E. coli 5359 strain was unable to grow in VB medium. The growth of E. coli 5359[pULAR11] was slow probably due to low efficiency of transcription of S. clavuligerus argH gene (see below). This gene is expressed from the S. clavuligerus argG promoter which is known to be functional in E. coli (Rodríguez-García et al., 1995).

Location of the Arginine Genes in Streptomyces coelicolor
The S. coelicolor arginine biosynthesis genes have been reported to be scattered in the genome by classical genetic methods (Redenbach et al., 1996), but the relation between the locus detected and the proteins encoded has not been further elucidated. To confirm whether the arginine genes were scattered a blot of an arg cluster using total RNA from 1,3) S. clavuligerus ATCC 27064 and 2,4) S. clavuligerus argR::aph. Protection of the corresponding transcript fragment was made with A) probe CJ B) GHP, C) Probe RG, D) Probe GH. RNA was isolated from cells grown in GSPG medium (1,2) or TSB medium (3,4). Controls: 5) Probe CJ. 6) tRNA from S. cerevisiae 7) Probe DR, 6) Probe GH. The arrows show the probes used and the protected bands.
In order to study the transcription of arginine biosynthesis of S. clavuligerus ATCC 27064 grown in GSPG medium for 24, 48, 72 and 96 h. The probes used were V (argR) (left panel) and VI (argG) (right panel). The standards correspond to RNA type II markers (Boehringer).

Figure 4. Time course of expression of the arginine genes as shown by Northern hybridization of total RNA from S. clavuligerus ATCC 27064 grown in GSPG medium for 24, 48, 72 and 96 h. The probes used were V (argR) (left panel) and VI (argG) (right panel). The standards correspond to RNA type II markers (Boehringer).

analyzed. Each cosmid was digested with NoI and electrophoresed in 0.8% agarose; the DNA fragments were blotted to Hybond NX membrane (Amersham) and hybridized with the different probes. Only cosmids L10 and L24 gave strong hybridization signals. A 3.2 kb NoI DNA fragment of cosmid L10 hybridized with probes internal to argJ, argD and argR indicating that these genes are contiguous in the S. coelicolor genome. A 4.9 kb NoI DNA band of cosmid L24 gave hybridization with the same probes suggesting that the genes argJ, D and R are probably located in the overlapping region between L10 and L24. Only the 4.9 kb DNA fragment of cosmid L24 gave hybridization with the argH probe. Cosmid 4G1, in which the argG gene has been located, did not gave positive hybridization with the argG probe as expected due to the major sequence differences between the argG genes of S. clavuligerus and S. coelicolor (Rodríguez-García et al., 1995).

Transcription of arg Genes in the Wild Type S. clavuligerus and in a S. clavuligerus argR-Disrupted Mutant
In order to study the transcription of arginine biosynthesis genes, a S. clavuligerus argR- disrupted mutant was obtained by transformation with plasmid pHZargR-. About 2% of the transformants were mutants disrupted in argR by double recombination and showed the phenotype kan\textsuperscript{R} ts\textsuperscript{R}. The mutation was confirmed by hybridization of total DNA of the wild type strain and the disrupted mutant with probes internal to argR and aph\text{tl} (kan\textsuperscript{R}) genes.

The transcription of the genes in the arginine cluster was studied by Northern hybridization and S1 mapping using total RNA of the wild type S. clavuligerus ATCC 27064 and the argR disrupted mutant S. clavuligerus argR::aph.

**Transcription of Genes for the Early Steps of the Pathway**
S1 mapping with the 464 nt CJ probe (Figure 3, lane 5) shows an hybridization band of 428 nt (Figure 3, lanes 2 and 4) with S. clavuligerus argR::aph RNA. This band, corresponding to the RNA fragment protected by the CJ probe, was found in cells grown in TSB medium (lane 4) and in GSPG medium (lane 2). S1 mapping experiments with total RNA from the wild type strain show a band of the same size but with weaker intensity (Figure 3A, lanes 1, 3 and insert). Quantification of the hybridization signal showed that the amount of RNA specific for argCJ is about 30 times higher in S. clavuligerus argR::aph than in the wild type strain. This result correlates well with the presence of an ArgR repressible Arg-box sequence upstream of argC (Rodríguez-García et al., 1997).

S1 mapping with the JB probe (286 nt) showed a single weak band of 272 nt corresponding to the homologous region in the probe and the protected RNA (not shown). Probe DR (449 nt) protected a RNA fragment of 373 nt (Figure 3B, lanes 1 to 4) which indicates that argD and argR are co-transcribed. Additionally, a weak band of protection of 157-158 nt was found in the same S1 mapping experiment suggesting the presence of an additional promoter located upstream of argR. These results suggest that the most probable transcription initiation site of the argR promoter is at an adenine (nt 4594 in Figure 2) located immediately downstream of argR. Therefore, it seems that the early genes argCJBDR are transcribed in a single mRNA of about 5 kb; additionally argR is transcribed from its own promoter. To confirm these results Northern analysis of total RNA was made using as probe a PCR-amplified DNA fragment corresponding to the whole argR gene (Probe V). A large and diffuse hybridization band that might corresponds to the degraded argCJBDR transcript was observed; additionally a strong hybridization band of about 0.6 kb (Figure 4A) was found corresponding to the argR monocistronic transcript. The intensity of the hybridizing bands was higher in RNA preparations from cells grown for 48 and 72 h.

When the signal of the different S1 protection bands was quantified, the maximal intensity corresponds to the polycistronic argCJBDR RNA from S. clavuligerus argR::aph grown in GSPG (Figure 3, panel A, lane 2, and panel B, lane 2). By comparison with the intensity of these bands (considered as 100% in Table 1 for the derepressed mutant) the intensity of the hybridization band of the polycistronic RNA from the wild type strain grown in GSPG medium was 3.5% (using probe CJ) and 2.8% (using probe DR) indicating an increase of about 30 times in mRNA levels in the derepressed mutant. In cells grown in TSB medium the intensity of the polycistronic transcript for the wild type strain are 1.5% (probe CJ) and 0.76% (probe DR) (Table 1; Figure 3, panel A, lane 1 and panel B, lane 3) with respect to the levels of the derepressed mutant in the GSPG medium and the data for S. clavuligerus argR::aph amounts to 66.5% (probe CJ) and 30.9% (probe DR) (Table 1). Comparison of the data in GSPG and TSB media (Table 1) suggests that the complex TSB medium probably contains enough arginine to partially repress the transcription of arginine biosynthesis genes. The intensity of the signal corresponding to the monocistronic argR transcript is relatively similar in all the strains and growth conditions used. Therefore it appears that the polycistronic mRNA accounts for most of the ArgR repressor protein.
Discussion

The presence of a cluster of seven contiguous genes for the biosynthesis of arginine in *S. clavuligerus* was unexpected since arg auxotrophic mutants map in four different loci in the *S. coelicolor* genome (Redenbach et al., 1996). The hybridization studies of the *S. coelicolor* DNA with our probes suggest that there are at least two clusters of arg genes in this model actinomycete. One cluster containing argC/J,D,R and B while argH is located close but not linked to the first subcluster. These results agree with those of Hindle et al. (1994) who found an argCJB cluster in *S. coelicolor* and with recent data on the genetic map of *S. coelicolor* (Sanger Center Sequencing Group).

In addition, the unstable argG of *S. coelicolor* maps near one of the ends of the *S. coelicolor* linear chromosome (Redenbach et al., 1996) at difference of what occurs in *S. clavuligerus* where argG forms part of the arginine cluster as reported in this article. Unstable argG genes are also present in *Streptomyces lavendulae*, *Streptomyces lividans*, *Streptomyces coelicolor*, *Streptomyces scabies* and *Streptomyces alboniger*. There are two types of argG genes in *Streptomyces* (Rodríguez-Garcia et al., 1995) and it is likely that the stable argG genes of *Streptomyces* species might be located in a large arg cluster whereas the unstable argG gene is located in a separate location near the end of the linear genome. This would suggest that argG suffered a translocation in a line of *Streptomyces* ancestors but not in other group of this large genus.

The absence of an argF gene, encoding ornithine carbamoyltransferase (forming citrulline) in the arg cluster of *S. clavuligerus* is surprising; argF is not located in the vicinity, either upstream or downstream of the *S. clavuligerus* arg cluster although argF is in a central position in the arg cluster of mycobacteria. Attempts to clone this gene by complementation of argF mutants of *E. coli* were unsuccessful, perhaps due to the lack of expression of its promoter in *E. coli*.

Regulation of the expression of the arginine cluster in *S. clavuligerus* by the arginine level is exerted at the transcription level. Two canonical 18-nt Arg-boxes with 70 to 80% similarity to the consensus *Streptomyces*Arg-box (Rodríguez-García et al., 1997) occur upstream of the argC and argG genes but only one 18-nt sequence with 50% similarity to Arg-boxes, is present upstream of argF (nucleotides 4581-4598). Indeed formation of the monocistronic argR transcript appears to be weak and constitutive (Table 1). This is in agreement with the results of arginine regulation in *E. coli* in which the presence of two arginine-boxes exerts a cooperative effect resulting in a stronger binding of the regulatory ArgR protein. Binding of the *B. subtilis* AhrC arginine repressor to the arg-boxes of argC has been reported previously (Rodriguez-Garcia et al., 1997). Expression of the arginine biosynthesis genes in the arginine-R-disrupted mutant is in the order of 10 to 44-fold that of the wild type strain (depending on the medium) (Table I). Arg-boxes are also present upstream of argC (Hindle et al., 1994), argG (Rodríguez-García et al., 1995) and argH (Sanger Center Sequencing Group) of *S. coelicolor* suggesting a similar type of regulation.

Our results are compatible with the co-transcription of the argG and argH genes. However the differences in intensity between the 3.3 kb and the 1.5 kb band obtained by Northern hybridization with the argG probe suggest that

### Table 1. Relative Percentage of Expression of the Early and Late arg Genes as Shown by S1 Protection Studies

<table>
<thead>
<tr>
<th>Strain</th>
<th>Transcription Increase* (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. clavuligerus</em> 27064</td>
<td><em>S. clavuligerus</em> argR::aph</td>
</tr>
<tr>
<td>TSB</td>
<td>GSPG</td>
</tr>
<tr>
<td>Probe CJ mRNA argCJBDR</td>
<td>1.5</td>
</tr>
<tr>
<td>Probe DR mRNA argCJBDR</td>
<td>0.7</td>
</tr>
<tr>
<td>mRNA argF</td>
<td>0.2</td>
</tr>
<tr>
<td>Probe GH mRNA argGH</td>
<td>3.0</td>
</tr>
</tbody>
</table>

* 1 60,959 net counts
* 2 906,824 net counts
* 3 36,095 net counts

* The expression increase is the ratio of mRNA expression of *S. clavuligerus* argR::aph in relation to the wild type strain.
the inverted repeat sequence existing 22 nt downstream of the argG TGA stop codon, which might form a stem and loop structure (Rodríguez-García et al., 1995), acts as a terminator (attenuator) and therefore only a small percentage of the RNA polymerase-initiated transcript are able to readthrough to the argH gene. This is an interesting example of differential expression of clustered genes in Streptomyces, that seems to occur also in long polycistronic transcript of antibiotic biosynthesis genes (Enguita et al., 1998).

Experimental Procedures

Bacterial Strains and Plasmids

The wild type S. clavuligerus ATCC 27064 was used as source of DNA. E. coli auxotroph strains XSlD2 (argE) and XB25 (argB) were kindly provided by S. Baumberg (Leeds University, UK). Strains E. coli CG3539 (argH) and E. coli CG5358 (argD) were obtained from the E. coli Genetic Stock Center.

Plasmids pULM31, pULAR1 and pULAR11 were constructed by Ludovice et al. (1992) and Rodriguez-Garcia et al. (1995). Plasmid pULD contains in pBSK (+) a 1.38 kb DNA fragment carrying the argD gene. Plasmid pULB contains the argB gene obtained by PCR in the EcoRV site of pULMA (Rodriguez-Garcia et al., 1995), downstream of the argC promoter. Plasmids pULJ1 and pULJ2 are clones used for the sequencing of the 6.1 kb Sau3A1 DNA fragment; they contain in pBSK (+) a 1.2 kb DNA insert containing the complete argU gene.

Culture Conditions

E. coli auxotrophy complementation was tested in VB medium (Smith and Yanofsky, 1962). Streptomyces strains were grown from mycelium stock kept in glycerol (20%) at -75°C. One milliliter of the frozen mycelium was used to seed a 100 ml TSB (3% trypticase-soy) culture. The TSB culture kept in glycerol (20%) at -75ºC. One milliliter of the frozen mycelium was used to inoculate 500 ml trippled baffled flasks which were centrifuged and used to inoculate 500 ml trippled baffled flasks which were grown for 36 h, the optical density was adjusted to O.D. 5.0 and 5 ml used to seed a 100 ml TSB (3% trypticase-soy) culture. The TSB culture was grown overnight. The S1 reaction sample (4 µl DNA sample plus 6 µl carrier buffer) was electrophoresed in 6% urea 7M. As size control the sequencing tRNA from Saccharomyces cerevisiae (type X-SA, Sigma) was utilized as negative control. The hybridization reaction was done at 67ºC overnight. The S1 reaction sample (4 µl DNA sample plus 6 µl carrier buffer) was electrophoresed in 6% urea 7M. As size control the sequencing reactions from M13mp18 were used. Quantification of the hybridization signals was done by densitometry using an Electronic Autoradiography Instant Image (Packard Ins, Meriden, USA).

RNA Isolation

RNA in the mycelium from 100 ml of a 24 h (TSB medium) or 48 h culture (GSPG medium) was extracted as described before (Pérez-Redondo et al., 1998) except that prior to the RNA purification, most of the DNA was specifically precipitated from the aqueous phase using isopropanol (v/v).

Probes Used in the S1 Analysis

Probe CJ: A 549 nt nucleotide fragment was amplified by PCR using as template the Smal-Sal DNA fragment corresponding to S. clavuligerus argR intergenic region and the oligonucleotides 1J: 5' -GGGCTGGCGTATCTTCCTT-3' and 1F: 5' -GGGCGTATCTTCCTT-3' that protrudes 5’ end, non-susceptible to labelling with polynucleotide kinase. This probe has 36 nt of heterologous DNA [corresponding to pBSK (+)].

DNA Manipulation and Transformation

Plasmid pHZargR-K (a pHZ1351-derived vector) contains the aphil gene inserted as a blunt-ended fragment into the BstXI site of the argR gene. S. clavuligerus was transformed with pHZargR-K to obtain the disrupted mutant S. clavuligerus argR::aph. E. coli strains were transformed according to Hanahan (1983). All DNA manipulations including PCR studies and Northern hybridization were made following standard procedures (Sambrook et al., 1989; Hopwood et al., 1985). S1 mapping was performed essentially as described by Sambrook et al. (1989). Total RNA from S. clavuligerus ATCC 27064 (200 µg) or S. clavuligerus argR::aph (100 µg) was used and 100-200 µg RNA from Saccharomyces cerevisiae (type X-SA, Sigma) was utilized as negative control. The hybridization reaction was done at 67ºC overnight. The S1 reaction sample (4 µl DNA sample plus 6 µl carrier buffer) was electrophoresed in 6% urea 7M. As size control the sequencing reactions from M13mp18 were used. Quantification of the hybridization signals was done by densitometry using an Electronic Autoradiography Instant Image (Packard Ins, Meriden, USA).

RNA isolation

RNA in the mycelium from 100 ml of a 24 h (TSB medium) or 48 h culture (GSPG medium) was extracted as described before (Pérez-Redondo et al., 1998) except that prior to the RNA purification, most of the DNA was specifically precipitated from the aqueous phase using isopropanol (v/v).

Probes Used in the S1 Analysis

Probe CJ: A 626 nucleotide fragment was amplified by PCR using as template the Smal-Sal DNA fragment corresponding to S. clavuligerus argC intergenic region and the oligonucleotides 1J: 5' -GGGCTGGCGTATCTTCCTT-3' and reverse M13mp18 (20-20) as primers. The 626 nt amplified region was treated with BstXI to obtain a 464 nt fragment with a protruding 5’ end, non-susceptible to labelling with polynucleotide kinase. This probe has 36 nt of heterologous DNA [corresponding to pBSK (+)].

Probe JB: The oligonucleotides 1B: 5' -CTTGGGGAGGGCGTTGTTCTT-3' and reverse M13mp18 were used to amplify by PCR a DNA template (SalI-NcoI 349 nt DNA fragment corresponding to the S. clavuligerus argJB intergenic region) subcloned in pBSK (+). The 349 nt amplified region was digested with KpnI giving a 290 nt probe that contains 21 nt of heterologous DNA [corresponding to pBSK (+)].

Figure 5. Organization of arginine biosynthesis gene clusters in different Gram positive and Gram negative bacteria and Archaebacteria. Note the different arrangement of the arg genes in S. clavuligerus and S. coelicolor (S. coelicolor StL24 cosmid sequence at the Sanger Center).
Prote DR: An oligonucleotide 1R: 5'-ACGCTAGTCGTTGTCG-3'
corresponding to the non-coding strand of argR was purified by PAGE and
10 pmol were labelled at its 5' end with [γ-32P] ATP (>185 TBq/mmol,
Amersham Ltd., England) and 8 units of T4 polynucleotide kinase (MBI
Fermentas, Lithuania). The labelled oligonucleotide was purified by
precipitation with ammonium acetate using (20 µg) as carrier.

Prote RQ: The oligonucleotide 1R: 5'-GTTGCTGAATTCGGCTGGTCGGCATCAAGT-3',
in which the nt in italics correspond to pBSSK(+) (non
homologue DNA) and 2G: 5'-CTTATCCCGAGGGCAT-3' were used to
amplify from plasmid pUL118 (Rodríguez-Garcia et al., 1995) a 649 nt
DNA fragment from Bacillus subtilis digested with EcoRI.

Probe GH: A 561 nt DNA probe with 11 nt non-homologous nucleotides at
the 5'-end of the non-labelled strand was amplify by PCR using
the oligonucleotides 1G: 5'-GTTGCTGAATTCGGCTGGTCGGCATCAAGT-3'
and 2H: 5'-GAACCGCTCCGCCCAGAG-3'.

All the probes were purified from 1.5% agarose gels using the Quiaex
Gel Extraction Kit (Qiagen, Germany) and denatured (except probe DR),
by incubation for 5 min at 65°C in the presence of NaOH (final concentration
0.1 M). The probes were precipitated with sodium acetate pH 5.2:ethanol
in the presence of glucose (10 µg) as carrier, suspended in reaction buffer
(Sambrook et al., 1989) and labelled with [γ-32P] ATP and 14 units of T4
polynucleotide kinase. After 50 min at 37°C the enzyme was inactivated by
heating and the probes were purified by precipitation with ammonium
acetate:ethanol. Additionally, probes I to VII (Figure 2) were used for low
resolution scanning with a filter and autoradiography.

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