Cloning and expression in *Escherichia coli* of a *Streptomyces coelicolor* A3(2) argCJB gene cluster

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From a partial Sau3AI library of *Streptomyces coelicolor* A3(2) DNA in pIJ916, two hybrid plasmids pGX1 and pGX2 were isolated that complemented *S. coelicolor* A3(2) or *S. lividans* arginine auxotrophs. Subcloning DNA from pGX1 in the *Escherichia coli* expression vector pRK9 containing the *Serratia marcescens* trp promoter gave rise to one plasmid, pZC2, that complemented *E. coli* argB, C, E and H auxotrophs, and another, pZC1, that complemented only the first three. The plasmids were markedly unstable in the various complemented hosts, to varying extents; pZC1 was characterized further as providing the stablest host/plasmid combinations. *In vitro* deletion of part of the vector’s trp promoter did not affect complementation of the argB and C auxotrophs, implying that the *S. coelicolor* A3(2) arg genes may be expressed from their own promoter. The trp promoter-less plasmids included isolates, such as pZC177, that had suffered extensive further deletion without loss of complementing ability. Extracts of an *E. coli* argE auxotroph carrying pZC177 showed ornithine acetyltransferase activity, indicating that the complementing gene is of the argJ type. The complementation properties of *in vitro* deletion derivatives of pZC177 indicated the gene order argC-J-B. Part of argC and the upstream region were sequenced; an ORF was identified whose predicted product showed appreciable homology with the *E. coli* and *Bacillus subtilis* ArgC polypeptide. Upstream of this ORF a consensus-type promoter and ribosome binding site could be discerned; overlapping its promoter was a sequence with homology to arginine operators in these two other organisms. An *in vitro* frameshift in argC had a polar effect on expression in *E. coli* of argJ and B, suggesting that the three genes are transcribed in the same direction, possibly as an operon.

Keywords: *Streptomyces coelicolor*, argCJB gene cluster, cloning, expression, arginine auxotrophy

INTRODUCTION

Studies on the molecular genetics of streptomycetes have so far largely concentrated on their uniquely varied secondary metabolism. Primary metabolic systems, in contrast, whether catabolic, anabolic or anaplerotic, have received less attention, particularly from the viewpoint of regulation. This is perhaps surprising, since secondary metabolites are necessarily synthesized from primary metabolite precursors whose pool sizes and rates of formation and breakdown are likely to influence secondary metabolite yields. Catabolic systems that have been studied in detail include those for glycerol in *Streptomyces coelicolor* A3(2) (Smith & Chater, 1988a, b) and galactose in *S. lividans* (Adams et al., 1988). Other amino acid pathways that have been examined in some detail are histidine biosynthesis in *S. coelicolor* A3(2) (Limauro et al., 1990) and proline synthesis, catabolism and transport and tryptophan biosynthesis (Hood et al., 1992). There is insufficient information, however, to tell
how the range of control devices (Baumberg, 1981) in streptomycetes compares to those of the enterobacteria or bacilli; or whether either in catabolic or anabolic systems there occur controls of gene expression analogous or even homologous to those found in other groups.

We have embarked on a study of arginine biosynthesis and catabolism in the model streptomycete *S. coelicolor* A3(2) to gain answers to such questions in this system, following on from work on the *Bacillus subtilis* system (Czaplewski *et al.*, 1992, and references therein). The first step was to clone genes of arginine biosynthesis. This could in principle be done by complementation of streptomycete auxotrophs, since arginine auxotrophic markers argA1, argB2 and argC4 are available in *S. coelicolor* A3(2) and argA in *S. lividans*. However, the only information available about these auxotrophs is their response to the arginine precursors ornithine and citrulline, leaving the biochemical lesions uncertain (see Fig. 1 for the arginine biosynthetic pathway); the *S. coelicolor* A3(2) locus designations argA, B and C (Hopwood *et al.*, 1985) therefore do not correspond to steps in the pathway as denoted in Fig. 1. The argA1, C4 and argA8 mutants respond to ornithine or citrulline, whilst the argB2 mutant responds only to arginine. As regards the map locations of arginine biosynthesis genes, the three *S. coelicolor* A3(2) mutant alleles are shown as mapping close together on the linkage map (Hopwood *et al.*, 1985). A further piece of information relates to the argG gene encoding argininosuccinate synthetase, which may be deleted in a variety of streptomycetes as part of vast deletions (sometimes of 2 Mbp), sometimes also associated with large amplifications (Schrempf, 1991; Altenbuchner & Eichenseer, 1991; Cullum *et al.*, 1991; Piendl *et al.*, 1991). As no other arginine biosynthesis gene has been unequivocally shown to be deleted in such cases, it seems likely that argG is located separately from other genes of arginine biosynthesis; an implication is that the gene affected in argB2 is argH, encoding argininosuccinate lyase. The *S. coelicolor* A3(2) argG gene has been cloned and sequenced (Ishihara *et al.*, 1985, 1991), as has that from *S. lavendulae* (Ogawara *et al.*, 1993).

As indicated in Fig. 1, there are two different forms of the pathway between glutamate and ornithine (Cunin *et al.*, 1986). In *Escherichia coli* and other enterobacteria, there are separate steps for synthesis of N-acetylglutamate from glutamate and acetyl-CoA, catalysed by N-acetylglutamate synthase (encoded by *argA*), and cleavage of N-acetylornithine to ornithine and acetate, catalysed by acetylornithinase (encoded by *argE*). In many groups, including pseudomonads and, as recently shown, bacilli (Sakanyan *et al.*, 1992, 1993; O'Reilly *et al.*, 1994), there is an ornithine acetyltransferase (whose gene is conventionally termed *argJ*) which catalyses the transfer of an acetyl group from N-acetylornithine to glutamate, the ArgA enzyme having only an anaplerotic role. An early report (Udaka, 1966), so far unconfirmed, suggested that *S. griseus* also possesses an ArgJ activity. The situation in *S. coelicolor* A3(2), whilst likely to resemble that in *S. griseus*, has not been investigated.

We describe here the cloning of *S. coelicolor* A3(2) genes of arginine biosynthesis and their introduction into defined *E. coli* auxotrophs to identify them precisely and map them with respect to each other, an approach used successfully in cloning arginine biosynthesis genes of *Bacillus subtilis* (Mountain *et al.*, 1986). We also report part of the sequence of one gene, *argC*, and its upstream region. In parallel with this work, Ludovicz *et al.* (1992) have reported the cloning, sequencing and determination of the transcription startpoint of *argC* from *S. clavuligerus*, in which organism the ornithine/arginine pathway provides precursors for clavulanic acid biosynthesis. These authors also showed linkage to *argC* of an *S. clavuligerus*

\textbf{METHODS}

\textbf{Bacterial strains and plasmids.} \textit{S. coelicolor} A3(2) prototroph M145 and arginine auxotrophs M124 (argA1), J12281 (argB2) and J12345 (argC44), and \textit{S. lividans} J11674 (argE8), as well as the SCP2*-based plasmid pJ1916, were provided by D. A. Hopwood and colleagues, John Innes Institute. The \textit{E. coli} strains all derive from this laboratory; HsdS\textsuperscript{-} arginine auxotrophs XA4 (argA4), XB25 (argB), XC33 (argC), XS1D2 (argE), XF (argF), XG31 (argG), XH11 (argH), XJ10 (carA) and XJeF8 (carB) are described in Mountain \textit{et al.} (1984), and 6P in Breitser & Baumberg (1976). The \textit{E. coli} plasmid vectors, also this laboratory's stocks, were pRK9 (Lim \textit{et al.}, 1989) and pUC18 (Messing & Vieira, 1982). The prototrophic \textit{Neospora crassa} strain 74R23-1A was kindly provided by A. Radford (Department of Genetics, University of Leeds).

\textbf{Media and growth conditions.} Media for \textit{E. coli} were as in Maniatis \textit{et al.} (1982) except for minimal media which were as in Mountain \textit{et al.} (1984). 2TY Amp and MinAmp refer to nutrient (2TY) or minimal media used for plasmid marker selection with ampicillin at 50 \textmu g ml\textsuperscript{-1}; MinAmp was always supplemented with any auxotrophic requirements other than arginine, in particular the disodium succinate (at 0.5 \%) needed by strain XS1D2 which carries a ppc-argE deletion. \textit{E. coli} strains were grown at 37 °C except where an arginine auxotroph was being complemented, when growth was at 30 °C. Media and growth conditions for \textit{Streptomyces} strains were as in Hopwood \textit{et al.} (1985).

\textbf{Enzyme assays.} Ornithine acetyltransferase was assayed by the method of Denes (1970). N-acetylornithinase was assayed by the method of Vogel & Bonner (1956). Protein was estimated as described by Bradford (1976).

\textbf{Isolation, restriction and ligation of DNA.} Plasmid DNA was isolated from \textit{E. coli} by the method of Birnboim & Doly (1979). Chromosomal DNA was isolated from \textit{S. coelicolor} A3(2) as in Hopwood \textit{et al.} (1985); plasmid DNA was isolated from \textit{Streptomyces} strains either according to Hopwood \textit{et al.} (1985) or by the method of Birnboim & Doly (1979). Restriction endonucleases, DNA ligase, mung bean single-strand nuclease and Klenow fragment of DNA polymerase were obtained from standard suppliers and used according to makers' recommendations.

\textbf{Techniques.} Gel electrophoresis, elution of DNA fragments from gels, preparation of radioisotope-labelled probes by nick-translation, and Southern blot hybridization were carried out essentially as in Maniatis \textit{et al.} (1982). Bacterial transformation was carried out according to Maniatis \textit{et al.} (1982) (E. coli) or Hopwood \textit{et al.} (1985) (Streptomyces strains).

\textbf{DNA sequencing.} The SaI(0)-Smal(1) fragment of pZ1H1801 and the ProII(1)-Smal(1) fragment of pZC1773 (see Fig. 3a) were each cloned into sequencing vectors M13mp8 and M13mp19 (Norrander \textit{et al.}, 1983). Sequencing was carried out with the Pharmacia T7 Polymerase Sequencing Kit according to the maker's instructions. To permit sequencing across the \textit{SalI} site and to the beginning of the \textit{SalI}-\textit{SalI} fragment, two additional 17-mer oligonucleotide primers (provided by the Biotechnology Unit, University of Leeds) were employed; these corresponded to bases 272–288 (strand shown) and 324–340 (complementary to strand shown) of Fig. 5.

\textbf{RESULTS}

\textbf{Cloning of \textit{S. coelicolor} A3(2) arginine biosynthesis genes in streptomycetes and their subcloning in \textit{E. coli} by complementation of arginine auxotrophs}

A gene library of the prototrophic \textit{S. coelicolor} A3(2) strain M145 was produced by partially digesting total DNA with \textit{Sa}a3AI and ligating fragments of size 8 kbp or above into the \textit{BgII} site of the SCP2*-based vector pJ1916 (Hopwood \textit{et al.}, 1985). The resulting hybrids were transformed into the \textit{S. coelicolor} A3(2) arginine auxotrophs M124, J12281 and J12345, and \textit{S. lividans} J11674, initially with selection for thiostrepton resistance; transformants were then replica plated on minimal medium without arginine to detect complementation. In this way, two plasmids, pGX1 and pGX2, were obtained that complemented streptomycete arginine auxotrophs; pGX1 complemented all four, pGX2 all except J12345, from which it may be concluded that pGX1 carries \textit{argH} (see Introduction), whilst pGX2 does not. Single and double digests with a number of restriction endonucleases (data not shown) yielded the restriction maps of the two plasmids shown in Fig. 2. They have inserts of 17.2 kbp and 129 kbp, respectively, with an overlap of about 10 kbp. The orientation of insert DNA in pGX1 with respect to the vector is opposite to that in pGX2.

We proceeded by subcloning DNA from pGX1 in an \textit{E. coli} vector. It is known that streptomycete genes frequently express at a low level or not at all from their own promoters in \textit{E. coli} (see discussion in Lim \textit{et al.}, 1989). We therefore used the expression vector pRK9 which has been used successfully before for a similar purpose, the subcloning of the \textit{S. griseus} \textit{aphD} gene (Lim \textit{et al.}, 1989); it is essentially pBR322 with the \textit{EcoR1–BamH1} fragment replaced by a fragment containing the \textit{Serratia marcescens} \textit{trp} promoter. pGX1 was partially digested with \textit{SalA}I and the fragments cloned into the \textit{BamH1} expression site of pRK9. The ligation mixture was transformed into the four \textit{E. coli} arg auxotrophs XA4, XB25, XC33 and XS1D2, carrying lesions in \textit{argA}, \textit{B}, \textit{C} and \textit{E}, respectively (Mountain \textit{et al.}, 1984). Transformants were selected on 2TY Amp and replicated on to MinAmp plates. Three \textit{Arg}\textsuperscript{+} isolates were obtained with XC33 as recipient and none with the other three strains; the plasmids in these three isolates were termed pZC1, pZC2 and pZC3. Preliminary restriction analysis of these plasmids (results not shown) indicated that they contained inserts of about 30, 31 and 17 kbp, respectively. Colonies of XC33 carrying pZC1 and pZC2 grew to reasonable size in 2–3 d, whilst those with pZC3 took about 5 d. For this reason, only pZC1 and pZC2 were studied further.

\textbf{Characteristics of pZC1 and pZC2: instability and complementation of further \textit{E. coli} auxotrophs}

When XC33(pZC1) or XC33(pZC2) were cultured in 2TY Amp, additional smaller plasmids were found, at
least some of which were unable on isolation to transform XC33 to Arg\(^+\) (results not shown). The smaller plasmids had presumably arisen by deletion and/or rearrangement as a result of which the presumed cloned \textit{S. coelicolor} A3(2) \textit{argC} gene either had been deleted completely or in part, or could no longer be expressed. On culture of XC33(pZC2) in MinAmp, pZC1 appeared to be stable. On culture of XC33(pZC2) in MinAmp, however, two plasmids could be discerned, one corresponding in size and restriction pattern to the original pZC2 and the other to a deletion derivative of size about 25 kbp; the latter, termed pZC201, could retransform XC33 to Arg\(^+\). On culture of XC33(pZC201) in MinAmp, the plasmid appeared to be stable.

Plasmids pZC1, pZC2 and pZC201 were used to transform others of the complete set of Hsd\(^-\) \textit{E. coli} \textit{arg} auxotrophs (Mountain \textit{et al.}, 1984), namely XA4 (\textit{argA}\(^-\)), XB25 (\textit{argB}\(^-\)), XS1D2 (\textit{ppc} \textit{argE}\(^-\)), XF (\textit{argF}\(^-\)), XG31 (\textit{argG}\(^-\)), XH11 (\textit{ppc} \textit{argECBH}\(^-\)), X190 (\textit{carA}\(^-\)) and X Jef8 (\textit{carB}\(^-\)). Transformants were again initially selected on 2TYAmp and replicated to MinAmp to check for Arg\(^+\). These tests showed that pZC1 could complement \textit{argB} and \textit{E} mutations, and pZC201 the \textit{argB} mutation, in addition to \textit{argC}, whilst pZC2 could complement \textit{argB}, \textit{E} and \textit{H} mutations. In all cases where complementation was observed, less than 100% of transformants selected on 2TYAmp gave Arg\(^+\) colonies when replicated on MinAmp. Plasmid DNA was isolated from the various

\textbf{Fig. 2.} Restriction maps and other details of plasmids pGX1, pGX2, pZC1, pZC177 and pZH1801. Stippled boxes, \textit{S. coelicolor} chromosomal DNA; bold-outline open boxes, SCP2\(^*-\)-based vector DNA; thin-outline open boxes, pBR322-based vector DNA. Hatched boxes refer to a sequence present in one copy in pGX1 and apparently in two copies, in inverse orientation (shown by hatching in opposite direction), in pZC1. Cross-squared boxes refer to a sequence apparently displaced in pZC1. For pZH1801, only the insert and immediately surrounding regions (comprising the lac promoter and polylinker) are shown. The scale of the restriction map is as shown. Abbreviations of restriction enzymes: B, \textit{BamHI}; Bg, \textit{BglII}; El, \textit{EcoRI}; EV, \textit{EcoRV}; Hp, \textit{HpaI}; P, \textit{PstI}; S3, \textit{Sau3AI}; Sa, \textit{SalI}; Sp, \textit{SphI}; Ss, \textit{SstI}; Xb, \textit{XbaI}. The numbering of sites is described in the text. Sites in pZC1 which are presumed to exist by similarity to the other plasmids but which have not actually been checked are indicated with lines but no lettering. The positions of the \textit{trp} and \textit{lac} promoters in pZC1 and pZH1801, respectively, are shown, arrowheads pointing in the direction of transcription. Approximate locations of the \textit{argCJB} genes are based on data shown in Fig. 3 and described in the text. The strategy for sequencing part of the insert in pZH1801 is shown together with the positions of internal sequencing primers used.
Amp<sup>R</sup> Arg<sup>+</sup> transformants, cut with PsvII or BglII, and electrophoresed. XB25(pZCl) yielded the equivalent of (in the sense of giving the same restriction pattern as) pZCl, and XB25(pZC2) the equivalent of pZC201; XS1D2(pZCl) yielded only deletion derivatives, XS1D2(pZC2) the equivalent of pZC201; and XH11(pZC2) yielded the equivalents of either pZC2 alone, pZC201 alone, or both. Whereas complementation of XB25, like that of XC33, gave colonies that reached reasonable size in 2-3 d, XS1D2(pZCl) or (pZC2) growing on MinAmp took 9-11 d to reach a similar size.

These results imply that pGX1 carries the <i>S. coelicolor</i> A3(2) analogues of argB, C, H and either E or J, the latter being presumably capable of complementing an *E. coli* argE mutation (see below). At the cost of not proceeding further with the *argH* gene, it was decided at this point to concentrate on pZCl as being the most stable of the plasmids.

**Restriction analysis of plasmid pZCl**

Single and double digests of pZCl with a variety of restriction enzymes (results not shown: see Hindle, 1990) provided the restriction map of this plasmid shown in Fig. 2. Comparison with the map of pGX1 suggests that a number of non-contiguous fragments of pGX1 had combined to give pZCl, as noted in the figure legend, and also that one region of pGX1 was present in duplicate in pZCl, in tandem but with inverse orientation: the SstI(1)-SstI(2) and SstI(3)-SstI(4) fragments of pZC1 probably correspond to SstI(1)-SstI(2) of pGX1.

**From what promoter are the arginine biosynthesis genes transcribed in pZCl?**

It is of interest to know whether in pZCl the *arg* genes are transcribed from their own promoter or from the *trp* promoter (or any other) in the vector. We have in previous cases (Hercomb et al., 1987; Lim et al., 1989) shown by deletion of part of a vector promoter that expression of cloned <i>S. coelicolor</i> genes was from that promoter and not from their own. pZCl, as with the hybrid plasmids described in Lim et al. (1989) (which are also pRK9 derivatives), has only single EcoRI and HpaI sites, the latter being within the *trp* promoter and the former just upstream of this. The sequence resulting from deletion of the DNA between these sites is predicted to be incapable of functioning as a consensus promoter (Lim et al., 1989). pZCl was therefore cleaved with EcoRI, 5' overhangs removed with mung bean nuclease, cleaved with HpaI and re-ligated the ligation mixture being transformed into XC33. Thirty-seven Amp<sup>R</sup> transformants were obtained on 2TY Amp. Of these, eight proved to be Arg<sup>+</sup> when tested on MinAmp but contained plasmids that had lost both sites; the Arg<sup>-</sup> transformants tested contained deleted versions of pZCl. On restriction analysis (not shown), three of the eight Arg<sup>+</sup> transformants were indistinguishable from pZCl except for the absence of the two restriction sites. Of the remainder, three appeared identical to each other but were derivatives of pZCl that had suffered large deletions, being only about 12 kbp in size. These three, however, like pZCl complemented the *argB* mutation of XB25 as well as the *argC* of XC33. With these smaller plasmids, it was found that 100% of transformants of either strain initially selected as Amp<sup>R</sup> on 2TY Amp proved also to be Arg<sup>+</sup> on MinAmp, an improvement on complementation with pZCl or pZC2 as noted above.

The simplest interpretation of the properties of the *trp* promoter deletions is that the cloned <i>S. coelicolor</i> A3(2) *arg* genes are not expressed from this promoter, and are either transcribed from some other vector promoter or from their own. Since pZCl is about 41 kbp, making it unwieldy to work with, all further work was carried out on one of the 12 kbp plasmids, termed pZC177. pZC177 also transforms the *argE* auxotroph XSlD2 to Arg<sup>+</sup>; XS1D2(pZC177) colonies grew on MinAmp within 2 d, considerably faster than XSlD2(pZCl).  

**Location of arginine biosynthesis genes within pZC177**

Single (sometimes partial) and double digests of pZC177 with a variety of restriction enzymes (results not shown: see Hindle, 1990) provided the restriction map of this plasmid shown in Figs 2 and 3(a). It may be noted that since the *PsvII(1)-SalI(1)* fragment of pZC177 is about the size expected for the small *PsvII-SalI* fragment of pRK9, the SalI(1) site of pZC177 might lie in vector DNA; in fact, it became apparent from DNA sequencing (see below) that this site lies within the <i>S. coelicolor</i> A3(2) insert DNA, the SalI site in the vector pRK9 evidently having been deleted. Comparison of the restriction maps of pZC177 and pGX1 (Fig. 2) suggested that the Spbl(1)-Spbl(2) fragment of pZC177 corresponded to the Spbl(3)-Spbl(4) fragment of pGX1; it seemed probable therefore that the SalI(1)-Spbl(2) region of pZC177 was colinear with the SstI(2)-Spbl(4) region of pGX1. This was checked by Southern blot hybridizations using the 1.28 kbp SalI(1)-Spbl(1), the 1.5 kbp Spbl(1)-BglII(1), the 2.67 kbp BglII(1)-BglII(2), and the 1.6 kbp BglII(1)-Spbl(2) fragments of pZC177 as probes against cleaved pGX1 and <i>S. coelicolor</i> M145 chromosomal DNA. The bands visible in the resulting autoradiographs (results not shown; see Hindle, 1990) were consistent with colinearity between the SalI(1)-Spbl(2) region of pZC177, the SstI(2)-PstI(1) region of pGX1, and a region of genomic DNA of <i>S. coelicolor</i> M145 equivalent to the latter.

To determine where within pZC177 lie the genes complementing the arginine auxotrophic mutations in XB25, XC33 and XS1D2, a number of derivatives were constructed by *in vitro* deletion. These were (Fig. 3a): pZC1771, in which the BglII(1)-BglII(2) fragment was deleted; pZC1772, in which the BglII(2)-BanHI(1) fragment was deleted; and pZC1773, in which the Spbl(1)-Spbl(2) fragment was deleted. An additional construct was pZC1774, in which the Spbl(1)-BglII(1) fragment was deleted from pZC1772. Plasmids pZC1771-4 were transformed into XB25, XC33 and XS1D2, and transformants tested for growth on minimal medium lacking arginine; Fig. 3a indicates whether or not complementation was
observed. The results clearly indicate a gene order C-E-B, where 'E' indicates that at this stage it cannot be said whether the complementing gene is an argE or an argF.

Further constructs made in part for sequencing (see below) were pZH1801 and pZH1901. In these, the 3.54 kbp SsrI(2)-BglII(1) fragment of pGX1 was ligated into SsrI/BamHI-cleaved pUC18 or 19, yielding pZH1801 (Figs 2 and 3a) and pZH1901. Restriction analysis of pZH1801 revealed a further SalI site between the SsrI(2) site and the SalI site corresponding to the SalI(1) site of pZC177. This new SalI site, which is absent from pZC177 and its derivatives, was termed SalI(0) (Figs 2 and 3a). Both pZH1801 and 1901 complemented XC33 and XSlD2; since this occurred with either orientation of the fragment with respect to the vector's lac promoter, expression in at least one orientation must rely on the insert's arg promoter, or an uncharacterized vector promoter.

argE or argF?

As noted in the Introduction, it has been reported by Udaka (1966) that S. griseus possesses an ornithine acetyltransferase activity and therefore probably uses the cyclic pathway of ornithine synthesis; it might be expected that S. coelicolor A3(2) will be similar. To test this, extracts of XSlD2 carrying pZC177, with, as controls, XSlD2 carrying the vector pRK9, the argE+ E. coli K-12 strain 6P (Bretscher & Baumberg, 1976) which possesses only argE, and an arg0 Neurospora crassa, which employs the cyclic pathway and therefore possesses argF (Davis, 1986), were assayed for their ability to convert acetylornithine to ornithine either in the presence of glutamate (which
Table 1. Specific activities of ornithine acetyltransferase and acetylornithinase in E. coli strains with and without cloned S. coelicolor genes and in N. crassa.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Enzyme activity [units (mg protein)^{-1}]</th>
<th>Ornithine acetyltransferase</th>
<th>Acetylornithinase</th>
</tr>
</thead>
<tbody>
<tr>
<td>XSD2(pRI9)</td>
<td>&lt; 5</td>
<td>&lt; 5</td>
<td></td>
</tr>
<tr>
<td>XSD2(pZC177)</td>
<td>15</td>
<td>&lt; 5</td>
<td></td>
</tr>
<tr>
<td>6P</td>
<td>&lt; 5</td>
<td>970</td>
<td></td>
</tr>
<tr>
<td>N. crassa 74R23-1A</td>
<td>1600</td>
<td>460</td>
<td></td>
</tr>
</tbody>
</table>

Cultures were grown in MinAmp (E. coli) or Vogel’s minimal medium (N. crassa). XSD2 cultures were supplemented with succinate and in the case of XSD2 with arginine (100 μg ml^{-1}) also. Specific activities are averages of two estimations made for different cultures.

measures both ArgE and ArgJ or in its absence (which measures only ArgE). The results are given in Table 1, from which it is seen that pZC177 confers on XSD2 an ornithine acetyltransferase activity, confirming the conclusion of Udaoka (1966). The low level of glutamate-independent activity found together with the ArgJ activity in the N. crassa extract may be non-specific, and seems to be general in microbial eukaryotes (see De Deken, 1963, for Saccharomyces cerevisiae). We can therefore say that the gene order in pZC177 is argC-J-B.

Sequence analysis of argC and its upstream region

The PvuI(1)-SphI(2) fragment from pZC1773 (as illustrated for pZC177 in Fig. 3a) and the SphI(0)-SphI(1) fragment from pZH1801 (Figs 2 and 3a) sequenced as in Methods, according to the strategy depicted in Fig. 2. The pZH1801 sequence, all of which was obtained on both strands, is shown in Fig. 4; the pZC1773 sequence showed 25 bp corresponding to pBR322 nucleotides 1027–1053 (Balbas et al., 1986), followed by nucleotide 246 of the sequence shown in Fig. 4. An ORF within the former was identified whose predicted translation product showed homology with the E. coli, B. subtilis and S. clavuligerus ArgC polyptides (Parsot et al., 1988; Smith et al., 1990; Ludovice et al., 1992). An alignment of these four and homologous sequences from *Saccharomyces cerevisiae* and an *Anaibaena* sp. are to be found in Ludovice et al., 1992), together with a detailed comparison of the sequences. Comparison of Figs 2 and 4 makes clear that the direction of transcription of *argC* is opposite to that directed by the trp promoter of the pRK9 vector, consistent with this gene being expressed from its own (or possibly an uncharacterized vector) promoter.

Direction of transcription of *argJ* and *argB*

To gain information on this point, a frameshift mutation was introduced into *argC* and the effects on ability of the downstream *argJ* and B to complement XSD2 and X825 determined. pZC1773 (Fig. 3a) was cleaved with SphI, the 5’ overhangs filled in with Klenow enzyme, and the blunt ends ligated to generate pZC1776. This treatment, as predicted, generated a new *PvuI* site at the original (and now destroyed) *SphI* site. The positions of restriction sites SaI(0), SaI(1) and *SphI*(1) referred to in Fig. 2 are indicated. Bases in bold type, 246–251 and 269–274, and dot-underlined bases 285–292 are putative −15/−10 promoter boxes and the ribosome binding site, respectively. Underlined bases, 245–262, indicate the 18 bp sequence with homology to known arginine operator sites of *E. coli* and *B. subtilis*.

![Fig. 4](https://example.com/fig4.png)
predicted in all cases. As regards complementation of the other E. coli auxotrophs, it is seen that pZC17731 behaved like pZC177, to which it should of course be identical. pZC17761 failed to complement XS1D2, though after 3 d incubation XS1D2(pZC17761) replicates on MinAmp began to show discrete Arg+ colonies within the area of the replica. It complemented XB25, but growth was slower than for XB25(pZC1773), colonies taking 3 d to reach the size achieved by XB25(pZC1773) colonies in 1.5 d. pZC17732 and pZC17762 failed to complement XS1D2 or XB25. These results imply that the cloned argC, J and B genes are transcribed in E. coli onto a single transcript, so that the frameshift mutation created in argC has a polar effect on expression of J and B. It seems likely therefore that the three genes have the same orientation. An alternative explanation which cannot as yet be completely ruled out is that ArgB and ArgJ polypeptides must interact with a functional ArgC polypeptide in order to function. However, such an obligatory interaction has not been found so far in any system, bacterial or otherwise.

DISCUSSION

We have shown above that plasmid pGX1 carries the S. coelicolor A3(2) argC, J (assumed to be such from enzyme data), B and H genes within 17.2 kbp of insert DNA, and that pZC177 carries the first three of these within about 5.8 kbp in the order C-J-B. Even if the S. coelicolor A3(2) argH is not clustered with the other three, this points to a gene arrangement different from any other group. In E. coli, there is an argECBH cluster which is divergently transcribed from a common promoter–operator region between E and C (Glansdorff, 1987). In Bacillus, in this an early cluster argCJBD-cpa-F and an unlinked late cluster argGH (Mountain et al., 1986; Sakanyan et al., 1992). As noted in the Introduction, argG seems to be located on its own in S. coelicolor A3(2) and probably other streptomycetes; the positions of argD and F are uncertain, as are those of genes determining carboxamyl phosphate synthesis. The data of Ludovice et al. (1992) suggest that in S. clavuligerus also, argC and J are linked (assuming that their gene complementing E. coli argE is in fact an argJ).

A frameshift mutation produced in vitro had a polar effect on expression of J and B, indicating that the latter two are transcribed in the same orientation as argC. It cannot of course be inferred that the three genes are co-transcribed in S. coelicolor A3(2); also, the suggestion that the three genes may be expressed in E. coli from their own promoter needs to be checked by comparative mapping of transcription starting points in the two organisms.

Our assay results of enzyme activities in extracts of XS1D2(pZC177) accord with those of Udaka (1966) in suggesting that streptomycetes possess an ornithine carbamoyltransferase, rather than a separate acetylglutamate synthase and acetylornithinase as in E. coli. That being so, it is not clear why the S. coelicolor A3(2) argJ gene fails to complement the E. coli argA mutant XA4, whereas the complete cloned B. subtilis argJ does so (and the 3' portion of the gene can complement E. coli argE but not argA: Mountain et al., 1986). The explanation may possibly lie in the nature of controls at the level of the various enzyme activities in the different species.

DNA sequencing indicates that the polypeptide product of S. coelicolor A3(2) argC is homologous to those of E. coli (Parso et al., 1988), B. subtilis (Smith et al., 1991) and S. clavuligerus (Ludovice et al., 1992); the pairwise percentage identities of the predicted ArgC polypeptides are S. coelicolor A3(2)/E. coli, 42%; S. coelicolor A3(2)/B. subtilis, 36%; and S. coelicolor/S. clavuligerus, 67% in the 248 residues we have so far sequenced. The sequence just upstream of S. coelicolor A3(2) argC shows a consensus promoter and putative ribosome binding site at appropriate positions, with a 6 bp inverted repeat ACGTGC/GCACGT, hyphenated by 4 bp, lying between the proposed -35 and -10 promoter boxes. Perhaps more interestingly, an 18 bp sequence ATTGAATTAAAGTGCGATC overlapping the -35 promoter box (see Fig. 4) shows 14/18 identity (blocks 2–9 and 15–18 being completely identical) to a sequence within the 'ARG box' (ArgR repressor binding site) of the E. coli argR gene (Lim et al., 1987; Glansdorff, 1987), and shows 8/10 identity to the sequence ATTAGAATTAA found within the B. subtilis argCO1 operator (Czapekiewski et al., 1992). Similarly, Ludovice et al. (1992) have pointed out a sequence TTGAAGTTACTGATTAGTTA-TAGTCA strongly resembling 'ARG boxes' in E. coli and B. subtilis. Recent work in our laboratory (A. Soutar, unpublished results) and that of P. Lisas (M. Ludovice, P. Carrachas & P. Lisas, personal communication) accords with the idea that these sequence similarities are not fortuitous.

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