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

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Fig. 1. Pathways of arginine biosynthesis in micro-organisms. Letters A–J refer to enzymes mediating each step, these being encoded by genes *argA*–*argJ*, respectively. For further information, see Cunin *et al.* (1986) and Glansdorff (1987).

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 *arg-8* 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 *arg-8* 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 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 bio-

synthesis; 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, Ludovice *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*

gene complementing an *E. coli* *argE* mutation, and that the arginine auxotrophy of *S. lividans* 1674 is due to an *argC* lesion. A preliminary account containing elements of both this work and that of Ludovice *et al.* (1992) appeared in Padilla *et al.* (1991).

METHODS

Bacterial strains and plasmids. *S. coelicolor* A3(2) prototroph M145 and arginine auxotrophs M124 (*argA1*), JI2281 (*argB2*) and JI2345 (*argC44*), and *S. lividans* JI1674 (*arg-8*), as well as the SCP2*-based plasmid vector pIJ916, were provided by D. A. Hopwood and colleagues, John Innes Institute. The *E. coli* strains all derive from this laboratory; Hsd⁻ arginine auxotrophs XA4 (*argA*), XB25 (*argB*), XC33 (*argC*), XS1D2 (*argE*), XF (*argF*), XG31 (*argG*), XH11 (*argH*), X190 (*carA*) and XJef8 (*carB*) are described in Mountain *et al.* (1984), and 6P in Bretscher & Baumberg (1976). The *E. coli* plasmid vectors, also this laboratory's stocks, were pRK9 (Lim *et al.*, 1989) and pUC18 (Messing & Vieira, 1982). The prototrophic *Neurospora crassa* strain 74R23-1A was kindly provided by A. Radford (Department of Genetics, University of Leeds).

Media and growth conditions. Media for *E. coli* were as in Maniatis *et al.* (1982) except for minimal media which were as in Mountain *et al.* (1984). 2TYAmp and MinAmp refer to nutrient (2TY) or minimal media used for plasmid marker selection with ampicillin at 50 µg ml⁻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. *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 *Streptomyces* strains were as in Hopwood *et al.* (1985).

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).

Isolation, restriction and ligation of DNA. Plasmid DNA was isolated from *E. coli* by the method of Birnboim & Doly (1979). Chromosomal DNA was isolated from *S. coelicolor* A3(2) as in Hopwood *et al.* (1985); plasmid DNA was isolated from *Streptomyces* strains either according to Hopwood *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.

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 *et al.* (1982). Bacterial transformation was carried out according to Maniatis *et al.* (1982) (*E. coli*) or Hopwood *et al.* (1985) (*Streptomyces* strains).

DNA sequencing. The *Sal*I(0)–*Sma*I(1) fragment of pZH1801 and the *Pvu*II(1)–*Sma*I(1) fragment of pZC1773 (see Fig. 3a) were each cloned into sequencing vectors M13mp8 and M13mp19 (Norrandet *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 *Sal*I(1) site and to the beginning of the *Sal*I(0)–*Sal*I(1) 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.

Computer-aided sequence analysis. This was carried out by use of the Leeds University OWL database and associated software (Akrigg *et al.*, 1988).

RESULTS

Cloning of *S. coelicolor* A3(2) arginine biosynthesis genes in streptomycetes and their subcloning in *E. coli* by complementation of arginine auxotrophs

A gene library of the prototrophic *S. coelicolor* A3(2) strain M145 was produced by partially digesting total DNA with *Sau*3AI and ligating fragments of size 8 kbp or above into the *Bgl*II site of the SCP2*-based vector pIJ916 (Hopwood *et al.*, 1985). The resulting hybrids were transformed into the *S. coelicolor* A3(2) arginine auxotrophs M124, JI2281 and JI2345, and *S. lividans* JI1674, 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 JI2345, from which it may be concluded that pGX1 carries *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 12.9 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 *E. coli* vector. It is known that streptomycete genes frequently express at a low level or not at all from their own promoters in *E. coli* (see discussion in Lim *et al.*, 1989). We therefore used the expression vector pRK9 which has been used successfully before for a similar purpose, the subcloning of the *S. griseus* *aphD* gene (Lim *et al.*, 1989); it is essentially pBR322 with the *Eco*RI–*Bam*HI fragment replaced by a fragment containing the *Serratia marcescens* *trp* promoter. pGX1 was partially digested with *Sau*3AI and the fragments cloned into the *Bam*HI expression site of pRK9. The ligation mixture was transformed into the four *E. coli* *arg* auxotrophs XA4, XB25, XC33 and XS1D2, carrying lesions in *argA*, *B*, *C* and *E*, respectively (Mountain *et al.*, 1984). Transformants were selected on 2TYAmp and replicated on to MinAmp plates. Three Arg⁺ 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.

Characteristics of pZC1 and pZC2: instability and complementation of further *E. coli* auxotrophs

When XC33(pZC1) or XC33(pZC2) were cultured in 2TYAmp, additional smaller plasmids were found, at

Fig. 2. Restriction maps and other details of plasmids pGX1, pGX2, pZC1, pZC177 and pZH1801. Stippled boxes, *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, *Bam*HI; Bg, *Bgl*II; El, *Eco*RI; EV, *Eco*RV; Hp, *Hpa*I; P, *Pst*I; S3, *Sau*3AI; Sa, *Sal*I; Sp, *Sph*I; Ss, *Sst*I; Xb, *Xba*I. 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 *trp* and *lac* promoters in pZC1 and pZH1801, respectively, are shown, arrowheads pointing in the direction of transcription. Approximate locations of the *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.

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 *S. coelicolor* A3(2) *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⁻ *E. coli* *arg* auxotrophs (Mountain *et al.*, 1984), namely XA4 (*argA*⁻), XB25 (*argB*⁻), XS1D2 (*ppc*⁻*argE*⁻), XF (*argFI*⁻), XG31 (*argG*⁻), XH11 (*ppc*⁻*argECBH*⁻), X190 (*carA*⁻) and XJef8 (*carB*⁻). Transformants were again initially selected on 2TYAmp and replicated to MinAmp to check for Arg⁺. These tests showed that pZC1 could complement *argB* and *E* mutations, and pZC201 the *argB* mutation, in addition to *argC*, whilst pZC2 could complement *argB*, *E* and *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

Amp^R Arg⁺ transformants, cut with *Pvu*II or *Bgl*II, and electrophoresed. XB25(pZC1) yielded the equivalent of (in the sense of giving the same restriction pattern as) pZC1, and XB25(pZC2) the equivalent of pZC201; XS1D2(pZC1) 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(pZC1) or (pZC2) growing on MinAmp took 9–11 d to reach a similar size.

These results imply that pGX1 carries the *S. coelicolor* 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 pZC1 as being the most stable of the plasmids.

Restriction analysis of plasmid pZC1

Single and double digests of pZC1 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 pZC1, as noted in the figure legend, and also that one region of pGX1 was present in duplicate in pZC1, in tandem but with inverse orientation: the *Sst*I(1)–*Sst*I(2) and *Sst*I(3)–*Sst*I(4) fragments of pZC1 probably correspond to *Sst*I(1)–*Sst*I(2) of pGX1.

From what promoter are the arginine biosynthesis genes transcribed in pZC1?

It is of interest to know whether in pZC1 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 *Streptomyces* genes was from that promoter and not from their own. pZC1, as with the hybrid plasmids described in Lim *et al.* (1989) which are also pRK9 derivatives, has only single *Eco*RI and *Hpa*I 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). pZC1 was therefore cleaved with *Eco*RI, 5' overhangs removed with mung bean nuclease, cleaved with *Hpa*I and re-ligated the ligation mixture being transformed into XC33. Thirty-seven Amp^R transformants were obtained on 2TYAmp. Of these, eight proved to be Arg⁺ when tested on MinAmp but contained plasmids that had lost both sites; the Arg[–] transformants tested contained deleted versions of pZC1. On restriction analysis (not shown), three of the eight Arg⁺ transformants were indistinguishable from pZC1 except for the absence of the two restriction sites. Of the remainder, three appeared identical to each other but were derivatives of pZC1 that had suffered large deletions, being only

about 12 kbp in size. These three, however, like pZC1 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^R on 2TYAmp proved also to be Arg⁺ on MinAmp, an improvement on complementation with pZC1 or pZC2 as noted above.

The simplest interpretation of the properties of the *trp* promoter deletions is that the cloned *S. coelicolor* A3(2) *arg* genes are not expressed from this promoter, and are either transcribed from some other vector promoter or from their own. Since pZC1 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 XS1D2 to Arg⁺; XS1D2(pZC177) colonies grew on MinAmp within 2 d, considerably faster than XS1D2(pZC1).

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 *Pvu*II(1)–*Sal*I(1) fragment of pZC177 is about the size expected for the small *Pvu*II–*Sal*I fragment of pRK9, the *Sal*I(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 *S. coelicolor* A3(2) insert DNA, the *Sal*I site in the vector pRK9 evidently having been deleted. Comparison of the restriction maps of pZC177 and pGX1 (Fig. 2) suggested that the *Sph*I(1)–*Sph*I(2) fragment of pZC177 corresponded to the *Sph*I(3)–*Sph*I(4) fragment of pGX1; it seemed probable therefore that the *Sal*I(1)–*Sph*I(2) region of pZC177 was colinear with the *Sst*I(2)–*Sph*I(4) region of pGX1. This was checked by Southern blot hybridizations using the 1.28 kbp *Sal*I(1)–*Sph*I(1), the 1.5 kbp *Sph*I(1)–*Bgl*II(1), the 2.67 kbp *Bgl*II(1)–*Bgl*II(2), and the 1.6 kbp *Bgl*II(2)–*Sph*I(2) fragments of pZC177 as probes against cleaved pGX1 and *S. coelicolor* M145 chromosomal DNA. The bands visible in the resulting autoradiographs (results not shown; see Hindle, 1990) were consistent with colinearity between the *Sal*I(1)–*Sph*I(2) region of pZC177, the *Sst*I(2)–*Pst*I(1) region of pGX1, and a region of genomic DNA of *S. coelicolor* 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 *Bgl*II(1)–*Bgl*II(2) fragment was deleted; pZC1772, in which the *Bgl*II(2)–*Bam*HI(1) fragment was deleted; and pZC1773, in which the *Sph*I(1)–*Sph*I(2) fragment was deleted. An additional construct was pZC1774, in which the *Sph*I(1)–*Bgl*II(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

Fig. 3. (a) Restriction maps of plasmids pZC177, pZC1771, pZC1772, pZC1773, pZC1774 and pZH1801, together with their patterns of complementation of *argC*, *E* and *B* *E. coli* auxotrophs. The scale of the restriction map is as shown. Abbreviations of restriction enzymes: B, *Bam*HI; Bg, *Bgl*II; El, *Eco*RI; P, *Pst*I; Pv, *Pvu*II; Sa, *Sal*I; Sm, *Sma*I; Sp, *Sph*I; Ss, *Sst*I. The numbering of sites is described in the text. (b) Restriction maps of plasmids pZC177, pZC1776, pZC17731, pZC17732, pZC17761 and pZC17762, together with their patterns of complementation of *argC*, *E* and *B* *E. coli* auxotrophs. The scale of the restriction map is as in Fig. 3(a) and the abbreviations of restriction enzymes are as described above. The numbering of sites is described in the text. The direction of cross-hatching of the Sp.1/Sp.2 fragment denotes that in pZC17731 and pZC17761 this is in the same orientation as is pZC177, whereas in pZC17732 and pZC17762 it is in the reverse orientation. The 'x' at the Sa.1 site in pZC1776, pZC17761 and pZC17762 denotes the frameshift mutation created by cutting, blunt-ending and religating (see text).

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 *argJ*.

Further constructs made in part for sequencing (see below) were pZH1801 and pZH1901. In these, the 3.54 kbp *Sst*I(2)-*Bgl*II(1) fragment of pGX1 was ligated into *Sst*I/*Bam*HI-cleaved pUC18 or 19, yielding pZH1801 (Figs 2 and 3a) and pZH1901. Restriction analysis of pZH1801 revealed a further *Sa*I site between the *Sst*I(2) site and the *Sa*I site corresponding to the *Sa*I(1) site of pZC177. This new *Sa*I site, which is absent from pZC177 and its derivatives, was termed *Sa*I(0) (Figs 2 and 3a). Both pZH1801 and 1901 complemented XC33 and XS1D2; 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 *argJ*?

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 XS1D2 carrying pZC177, with, as controls, XS1D2 carrying the vector pRK9, the *argE*⁺ *E. coli* K-12 strain 6P (Bretscher & Baumberg, 1976) which possesses only *argE*, and an *arg*⁺ *Neurospora crassa*, which employs the cyclic pathway and therefore possesses *argJ* (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*

Cultures were grown in MinAmp (*E. coli*) or Vogel's minimal medium (*N. crassa*). XS1D2 cultures were supplemented with succinate and in the case of XS1D2 with arginine (100 µg ml⁻¹) also. Specific activities are averages of two estimations made for different cultures.

Strain	Enzyme activity [units (mg protein) ⁻¹]	
	Ornithine acetyltransferase	Acetylornithinase
XS1D2(pRK9)	< 5	< 5
XS1D2(pZC177)	15	< 5
6P	< 5	970
<i>N. crassa</i> 74R23-1A	1600	460

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 XS1D2 an ornithine acetyltransferase activity, confirming the conclusion of Udaka (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 *PvuII*(1)–*SalI*(2) fragment from pZC1773 (as illustrated for pZC177 in Fig. 3a) and the *SalI*(0)–*SmaI*(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 1027–1003 (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 polypeptides (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 *Anabaena* 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 XS1D2 and XB25

Sa.0	10	20	30	40	50	60	70
	GTCGACGAACCCCTTCGCTCAGCGCGTCTGCGCCCTGCGGATACCGCTCGGTGTTCCCATGGCGAACCCAC						
	80	90	100	110	120	130	140
	AGGGGAAGCGGAGGGCGCACAGGACACGACGAGCGGGCGGCTGCCACGGCCTCAGCCCGACGACCA						
	150	160	170	180	190	200	210
	GCTCGACGATCCAGGCACGACAGCCACCGTGCCTGGAGTCCGGAAGCGGACGGCTAGCCGACG						
	220	230	240	250	260	270	280
	CCTCGGCGAGCGCTCGAGGTACCCCGCATCGCATTCGCATAAACGTCATCGGCACGTATAGTCATGCT						
	290	300	310	320	330	340	350
	CTCCAGGAGGAGTATTTCCATGGCGGTACGTGCGGCGGTGGCCGAGCGAGTGGGTATGCGGGCGGTGA						
		M	A	V	R	A	A
		V	A	G	A	S	G
		A	G	A	S	G	A
		G	A	S	G	A	G
	360	370	380	390	400	410	420
	GCTGCTGCGGCTGCTCTCTGACCCATCCGAGGTGAGATCGGCGCCCTGACCGGCAACTCCAACGCGGT						
	L	L	R	L	L	L	T
	H	P	E	V	E	I	G
	A	L	T	G	N	S	N
	A	G	A	S	G	A	G
	430	440	450	460	470	480	490
	CAGCGGCTGGGTGCGCTTCAGCCGATCTGCTGCCGTGGCCGACCGGTGCTGGAGGCGACCAACCCCG						
	Q	R	L	G	A	L	Q
	P	H	L	L	P	L	A
	D	R	V	L	E	A	T
	T	T	P				
	500	510	520	530	540	550	560
	AGGTGCTCGGCGTCAAGCGTCTCTCTCGCGTGCAGCGGCGAGTCCGCGCGGTTCGCGGAGCA						
	E	V	L	G	H	D	V
	V	F	L	A	L	P	H
	G	Q	S	A	A	V	A
	E	Q					
	Sa.1						
	570	580	590	600	610	620	630
	GCTCGGACCGGACGCTCTCTGCTGACATGGGCGCGGACTTCGGCTGAAGGACCGGTGACTGGGAG						
	L	G	P	D	V	L	V
	D	M	G	A	D	F	R
	L	K	D	A	G	D	W
	640	650	660	670	680	690	700
	CGGTCTACGGCTCCCGCACGCGGCGACCTGGCCCTACGGCTCCCGAATTCGGGTGCGCGCGCG						
	R	F	Y	G	S	P	H
	A	G	T	W	P	Y	G
	L	P	E	L	P	G	A
	R	A					
	710	720	730	740	750	760	770
	CGTGGAGGGGTCCAAGCGCATCGCGGTGCCCGGTGCTACCCGACCGCGTCTCCCTGGCCCTCTTCCC						
	A	L	E	G	S	K	R
	I	A	V	P	G	C	Y
	P	T	A	V	S	L	A
	L	F	P				
	780	790	800	810	820	830	840
	GGCGTACCGCGGTGCTGGCGGAGCCCGAGGCGGTGATCGTGGCGGCTCCGCGACCTCCGCGCGGGC						
	A	Y	A	A	S	L	A
	E	P	E	A	V	I	V
	A	A	S	G	T	S	G
	A	G					
	850	860	870	880	890	900	910
	AAGGCGGCAAGCGCACCTGCTGGGCGAGGTGATGGGTCCATGTCGCGTACCGCGTCCGCGCGG						
	K	A	A	K	P	H	L
	L	G	S	E	V	M	G
	S	M	S	P	Y	G	V
	G						
	920	930	940	950	960	970	980
	GOCACGCGCACACCCCGAGATGATCCAGAACCTCGGCGCGTCCGCGGAGCCCGTACCGTCTCTCTT						
	G	H	R	H	T	P	E
	M	I	Q	N	L	G	A
	V	A	G	E	P	V	T
	S	F					
	Sa.1						
	990	1000	1010	1020	1030	1040	
	CACTCGACCGCTCGGCGGATGCGGCGGCGATCCTCGCCACCTGCACGCGGAAGCGGAAGCCCGGG						
	T	P	T	L	A	P	M
	P	R	G	I	L	A	Y
	C	T	A	K	A	K	P
	G						

Fig. 4. DNA sequence of the *SalI*(0)–*SmaI*(1) fragment of pZH1801, with the predicted amino acid sequence encoded by an ORF. The positions of restriction sites *SalI*(0), *SalI*(1) and *SmaI*(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 –35/–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*.

determined. pZC1773 (Fig. 3a) was cleaved with *SalI*, the 5' overhangs filled in with Klenow enzyme, and the blunt ends religated to generate pZC1776. This treatment, as predicted, generated a new *PvuII* site at the original (and now destroyed) *SalI* site. The *SphI*(1)–*SphI*(2) fragment from pZC177 (deletion of which generates pZC1773) was now cloned back into pZC1776, and into pZC1773 as a control. In both cases, isolates were obtained with the *SphI* fragment cloned in both orientations; in pZC17731 and pZC17761, it was in the original orientation, and in pZC17732 and pZC17762 it was in the reverse orientation. The composition of the resulting plasmids are shown in Fig. 3b, together with their abilities to complement XC33, XS1D2 and XB25. Complementation of XC33 was as

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*, there is 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 carbamoyl 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 acetyltransferase, 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* (Parsot *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 ATTGCATAAACGTGCATC 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 ATTGAATTAA found within the *B. subtilis argCO1* operator (Czaplewski *et al.*, 1992). Similarly, Ludovice *et al.* (1992) have pointed out a sequence TTGATAAAGTGCAGTGATTTGTA-TAGTCA strongly resembling 'ARG boxes' in *E. coli* and *B. subtilis*. Recent work in our laboratory (A. Soutar, unpublished results) and that of P. Liras (M. Ludovice, P. Carrachas & P. Liras, personal communication) accords with the idea that these sequence similarities are not fortuitous.

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