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Resistance, regulatory and production genes for the antibiotic methylenomycin are clustered

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At least 17 kb of DNA from the large unisolatable *Streptomyces coelicolor* A3(2) plasmid SCP1 are concerned with methylenomycin biosynthesis. Mutational cloning analysis, using insert-directed integration of *att* site deleted phage vectors into an SCP1-containing host, provided evidence of two large transcription units, of at least 6.6 kb and 9.5 kb. At the leftmost apparent end of the larger (left-hand) transcription unit is a region apparently involved in negative regulation of methylenomycin biosynthesis: when fragments from this region were used to direct phage integration, marked overproduction of methylenomycin resulted. The methylenomycin resistance determinant is located at the rightmost end of this same transcription unit. Hybridisation analysis with 13 kb of the cloned *mmy* region showed that it was closely similar to a segment of pSV1, a plasmid that specifies methylenomycin biosynthesis in *S. violaceus-ruber* SANK 95570.

Key words: mutational cloning/phage vectors/plasmids/*Streptomyces* genetics/transcription mapping

Introduction

Two *Streptomyces* strains, *S. violaceus-ruber* SANK 95570 (Haneishi *et al.*, 1974a,1974b) and *S. coelicolor* A3(2) (Wright and Hopwood, 1976) produce the antibiotic methylenomycin. In both cases, the production and resistance genes are carried on transmissible plasmids: pSV1 in *S. violaceus-ruber* (Aguilar and Hopwood, 1982) and SCP1 in *S. coelicolor* (Kirby and Hopwood, 1977). There is no other clear example of plasmid specification of antibiotic production in *Streptomyces* (Hopwood, 1983). Neither pSV1 nor SCP1 could be purified in significant quantities, perhaps partially because both plasmids consist of considerably >100 kb of DNA (Hopwood *et al.*, 1979; Aguilar and Hopwood, 1982; Chater and Bruton, 1983), so that cloning of methylenomycin genes directly from isolated plasmid DNA was not possible. Chater and Bruton (1983) recognised a large number of SCP1 fragment-containing derivatives of the phage vector ϕC31 KC400 (Harris *et al.*, 1983), because the inserts allowed this *att* site deleted vector to lysogenise SCP1-containing streptomycetes. Nine of these phages inactivated methylenomycin production on integration, indicating that they contained inserts internal to transcription units for methylenomycin production (*mmy* DNA). At least three classes of mutagenic (!*mmy*!) fragments, totalling at least 7 kb, were represented in the nine clones.

Here we show by hybridisation that these fragments are closely linked to each other and to a previously cloned methylenomycin resistance gene, *mmr* (Bibb *et al.*, 1980; Chater and Bruton, 1983) and describe a simple procedure for shotgun cloning SCP1 sequences flanking the previously cloned fragments. Mutational cloning with fragments of the resulting clones has revealed that a continuous segment of >17 kb of DNA including two very large transcription units codes for methylenomycin production and resistance, and contains a regulatory gene.

Results

Close linkage of genes for methylenomycin production and resistance

To discover whether the *mmr*-containing fragment and representatives of three classes of !*mmy*! DNA fragments previously isolated (*Pst*I fragments A4.2, C2.18 and C8.7: Chater and Bruton, 1983) were closely linked, each was used as a ^{32}P-labelled probe in Southern blot analysis of various restriction digests of DNA from *S. parvulus* 2296 (Hopwood and Wright, 1973: SCP1 DNA is amplified in this strain; Chater and Bruton, 1983). Figure 1a shows the sizes of fragments hybridising strongly with each probe. Assuming that hybridisation of two probes to a band of a particular size reflects linkage of the two probe sequences in the original SCP1 DNA, then the four probe fragments all originated from one 14-kb region of SCP1, and the arrangement of Figure 1a is the only configuration consistent with all the hybridisation data and with the known locations of restriction sites in the probe fragments. The resistance gene *mmr* is located between biosynthetic genes.

The arrangement in Figure 1a predicts that in SCP1 DNA there should be one or more small *Pst*I fragments (totalling ~0.5 kb) separating the A4.2 and C8.7 fragments and containing sites for *Xho*I and *Bam*HI but not for *Sst*I or *Bgl*II; and that to the right of C8.7 there should be sites for *Sst*I, *Bam*HI and *Xho*I within ~1 kb. This was supported by earlier mutational cloning experiments (Chater and Bruton, 1983) done with size-fractionated (2–6 kb) *Pst*I-partially-digested DNA: two clones (C9.15 and A3.13) of four that contained the same 2.3-kb *Pst*I fragment as C8.7 contained additional, smaller, *Pst*I fragments (Figure 1a). The C9.15 additional fragment of 0.5 kb contained *Xho*I and *Bam*HI but not *Sst*I or *Bgl*II, sites, strongly suggesting that its location is between the A4.2 and C8.7 *Pst*I fragments. The A3.13 additional fragment, of 1 kb, contained *Xho*I, *Sst*I and *Bam*HI sites, compatible with its location in SCP1 being immediately to the right of the C8.7 *Pst*I fragment. The 1-kb fragment from A3.13 was subcloned into an *attP*-deleted ϕC31 vector (KC400: Figure 2) and generated Mmy$^-$ lysogens when used to direct prophage integration into an SCP1-containing recipient, confirming its relevance to methylenomycin production.

Taken together, the results in Figure 1a suggest that the cloned *Pst*I fragments to the right of *mmr* are contiguous and are part of a single *mmy* transcription unit of at least 6.5 kb.

Longer cloned sequences containing mmy DNA

The DNA of a !*mmy*! mutant generated by a KC400::!*mmy*! phage contains the *vph* gene of the vector adjacent to genomic *mmy* DNA. This makes it possible to clone this adjacent DNA in *E. coli* vectors of the pBR322 family, by the shotgun cloning of viomycin resistance (Figure 1b). Such experiments were done with DNA of J1507 lysogens carrying KC420 (containing !*mmy*-

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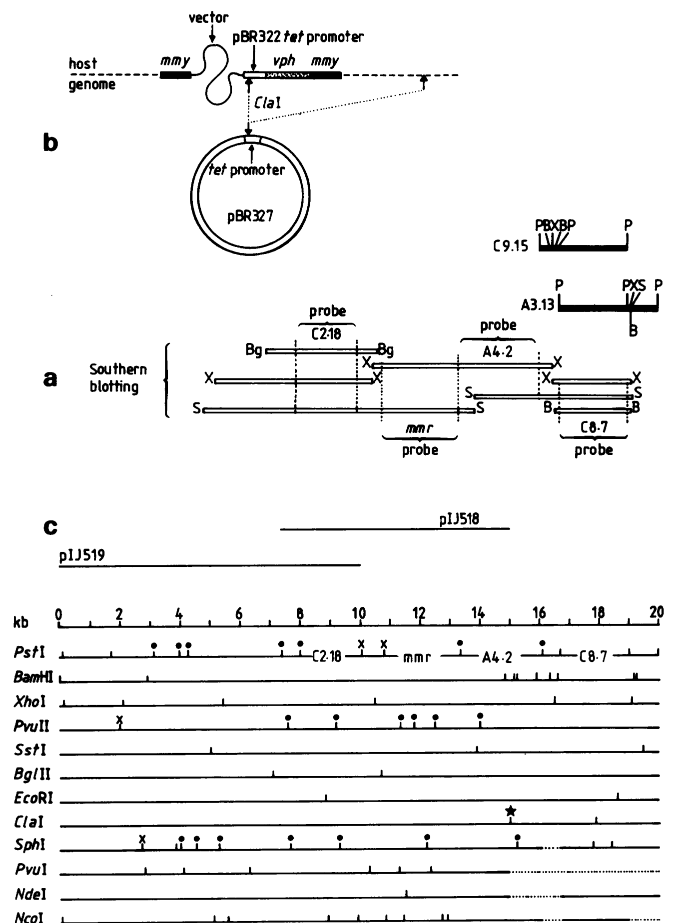

Fig. 1. (a) Demonstration by Southern hybridisation that independently cloned !*mmy*! and *mmr* fragments are closely linked in SCP1. Total DNA of *S. parvulus* 2296 (SCP1$^+$) was digested with *Bam*HI (B), *Bgl*II (Bg), *Pst*I (P), *Sst*I (S) or *Xho*I (X) and subjected to Southern hybridisation with nick-translated DNA from phages carrying the *Pst*I-generated !*mmy*! inserts C2.18, A4.2 or C8.7 or the *mmr*-containing *Pst*I insert MP2.7 (Chater and Bruton, 1983). The sizes of bands strongly hybridising to each probe are indicated by white boxes. These are arranged in the only sequence compatible with the observed data and with the known restriction maps of the probe fragments. The major hybridising fragments with *Pst*I digests were in each case identical in size to the probe fragment (indicated by vertial dotted lines). The restriction maps (heavy lines) of two other primary *Pst*I-generated !*mmy*! clones (C9.15 and A3.13, both containing the C8.7 fragment plus small extra fragments: Chater and Bruton, 1983) are aligned with the hybridisation map to show that the small fragments probably originated from these locations. **(b)** Cloning SCP1 sequences flanking !*mmy*! DNA. Total DNA from lysogens containing the vector ϕC31 KC400 integrated into *mmy* DNA (by insert-directed recombination) was cleaved in this example with *Cla*I and inserted into the *Cla*I site of pBR327. Ampicillin-resistant *E. coli* transformants obtained with the ligation mixture were replicated to nutrient agar (Difco) containing viomycin (50 μg/ml). Resistant colonies were obtained due to the expression of the *vph* gene from the reconstituted *tet* promoter. They contained SCP1 DNA extending through the originally cloned !*mmy*! fragment to the nearest adjacent *Cla*I site. Successful experiments were also carried out using the *Bam*HI site in the pBR327 *tet* gene, which is also present in the *tet* segment of the KC400 vector (see Results). **(c)** Restriction map of the cloned *mmy* region of SCP1. The locations of cloned *Pst*I segments used in **a**, and the extents of two clones (pIJ518, pIJ519) obtained as shown in **b**, are illustrated. The left-most ~2.5 kb of the map (derived from pIJ519) is not contiguous with the remainder of the map in SCP1 DNA (see Results). In the region from *Pvu*II site 2.0 to *Pst*I site 16.1, Southern blot analysis of *Pst*I, *Pvu*II and *Sph*I digests showed that sites marked with dots are present in both SCP1 and the *S. violaceus-ruber* plasmid pSV1, and those marked with crosses are absent from pSV1 (see Results). The *Cla*I site marked with an asterisk is protected in *E. coli* Dam$^+$ strains. Unmapped regions are shown as dotted lines.

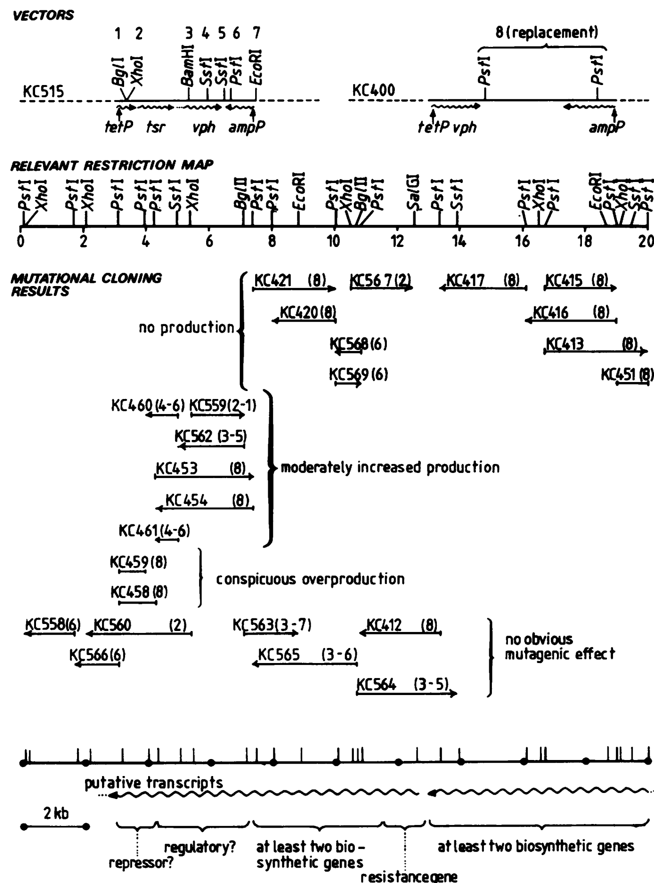

Fig. 2. Analysis of the *mmy* region by mutational cloning. The vectors used were KC515 (Rodicio *et al.*, 1985) or KC400 (Harris *et al.*, 1983) which are illustrated at the top of the figure. Known vector transcripts (wavy lines: Rodicio *et al.*, 1985) are illustrated in relation to the cloning sites used (numbered 1–8). The lines below the relevant restriction map of the *mmy* region show segments incorporated in different phages (KC numbers) with an indication of the vector sites used (numbers 1–8) and the orientation of the insert in relation to the vector's restriction map (arrows, where known). The inserts were used to direct the phages into the *mmy* region of *S. coelicolor* strain J1507 which carries SCP1 integrated into its chromosome (Chater and Bruton, 1983), and the resulting lysogens tested for methylenomycin production as in Figure 4. The interpretation of these and preceding results is summarised at the bottom of the figure.

C2.18!) and KC421 (the C9.12 clone of Chater and Bruton, 1983, containing the 2.05-kb C2.18 fragment in the opposite orientation, together with an additional small *Pst*I fragment). Thus, clones were isolated that carried SCP1 sequences extending leftwards or rightwards, respectively, from the 2.05-kb *Pst*I fragment common to KC420 and KC421. The numbers of *E. coli* transformants that were viomycin resistant were, with J1507 (KC421) DNA, 9/3589 (using *Cla*I) and 17/~15 000 (using *Bam*HI); and with J1507 (KC420) DNA, 6/~4400 (using *Bam*HI). Six to nine plasmids from each cloning experiment were identical as judged by *Bam*HI and *Pst*I digestion. The rightwards *Cla*I and *Bam*HI clones were closely similar to each other, suggesting that the nearest *Bam*HI and *Cla*I sites to the right of the 2.05-kb *Pst*I fragment were located close together. Further detailed restriction mapping (Figure 1c) was confined to one example (pIJ518) of a rightward *Cla*I clone and one (pIJ519) of a leftward *Bam*HI clone.

The pIJ518 map agreed well with the predictions from Southern hybridisation (Figure 1a): regions indistinguishable from the C2.18 fragment and (part of) A4.2 were on either side of a

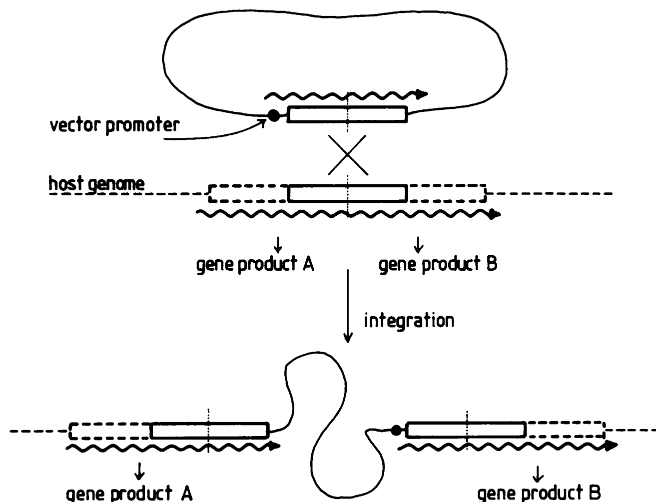

Fig. 3. A cloned fragment from within a polycistronic operon may be non-mutagenic when used to direct vector integration if it contains an inter-cistronic region (vertical dotted line) *and* if readthrough transcription from a vector promoter is at a suitable strength.

2.55-kb *Pst*I fragment identical to that containing *mmr*. Moreover, the latter fragment was separated from C2.18 by a previously unisolated 0.8-kb *Pst*I fragment containing sites for *Bgl*II and *Xho*I. The pIJ519 map contained, in addition to the C2.18 *Pst*I fragment, only 0.5 kb of DNA previously recognised in our studies: namely, a *Pst*I fragment identical to that originally cloned, along with the 2.05-kb C2.18 fragment, in KC421 (clone C9.12 of Chater and Bruton, 1983). Since KC421 was previously shown to be mutagenic, this 0.5-kb fragment must also be internal to the *mmy* transcription unit containing the 2.05-kb fragment.

The mmy regions of SCP1 and pSV1 are homologous

Hybridisation with pIJ518 and pIJ519 as probes was used to find out whether the clones were rearranged with respect to SCP1, and to estimate the degree of similarity of the *mmy* genes of SCP1 to those of the pSV1 plasmid of *S. violaceus-ruber* SANK 95570. The Southern blots used were of *Pst*I, *Pvu*II and *Sph*I digests of total DNA from *S. violaceus-ruber* (pSV1$^+$); *S. coelicolor* J1507 (containing an integrated copy of SCP1); J1507 (KC420) (the DNA sample from which pIJ519 was constructed); *S. parvulus* 2296 (SCP1$^+$); and of DNA of pIJ518 and pIJ519.

This experiment revealed that although the region from the *Pst*I site at 3.1 kb to that at 16.1 kb was indeed arranged as in SCP1, the leftmost 2.5 – 3 kb of pIJ519 (and therefore of all the left-ward *Bam*HI clones) was rearranged compared with the equivalent region of DNA from any of the test strains including J1507 (KC420). This implies that the change took place in *E. coli*, and that it occurred in each of six independent clones (all of which also contained an unexpected *Bam*HI site close to the rightmost end of the rearranged region). We have not investigated the nature of this rearrangement in detail. The unrearranged region was also very similar to that in pSV1: seven of nine *Pst*I sites, all of six *Pvu*II sites and all of seven *Sph*I sites were present in both pSV1 and SCP1 (sites not in common are marked with 'x' symbols in Figure 1c). Thus, considering only the 132 bp involved in these 22 restriction sites, and assuming that the changed sites involved single base changes, the two sets of sites (and presumably the whole region) differ at only 1.5% (2/132) of base pairs. There was no suggestion of significant insertions, deletions or rearrangements differentiating this region of the two plasmids.

The DNA of two ϕC31 KC400 derivatives (L2 and L5; Chater and Bruton, 1983) containing cloned SCP1 segments believed to be unrelated to *mmy* DNA was used to probe *S. violaceus-ruber Pst*I-digested DNA. The L5 probe gave no signal, and the L2 probe (containing two SCP1 *Pst*I fragments) gave only weak hybridisation, and to fragments of a different size (not shown). It thus appears that the *mmy* regions of pSV1 and SCP1 are particularly similar, indicating a possible relatively recent common origin for *mmy* DNA in the two plasmids, which are otherwise less closely related.

Mutational cloning analysis of the 20 kb of cloned SCP1 DNA containing mmy and mmr sequences

Various segments of pIJ518 and 519 (ranging from 0.75 to 3.4 kb) were subcloned into *attP*-deleted ϕC31 vectors, clones being recognised by their ability to direct integration of the vector into SCP1 DNA present in strain J1507. We expected, and found, that mutations affecting methylenomycin production would be generated by some of these integration events, providing information about the function and organisation of *mmy* DNA (Chater, 1983). The summarised results in Figure 2 are discussed in more detail below, using the kilobase co-ordinates of Figures 1 and 2.

Evidence for two major transcription units for structural mmy genes. Previous mutational cloning studies had shown that the *Pst*I fragment carrying *mmr* was not internal to an essential *mmy* transcription unit (Chater and Bruton, 1983), although Figure 1 shows that it is located between two *mmy* regions. This was supported by the wild-type production level of lysogens obtained with KC564, carrying the 10.5 – 13.9 kb segment. The interval between the two *mmy* regions was more closely defined by the finding that Mmy$^-$ mutants were generated by KC568 and KC569 (with the 10.0 – 10.8 kb segment in either orientation) and KC567 (carrying the 10.5 – 12.5 kb segment). The left-hand *mmy* transcription unit, previously known to include the 7.3 – 10.0 kb segment, thus extends at least from 7.3 to 12.5 kb, coming within 0.8 kb of the right-hand unit known (see above) to extend at least from 13.3 to 20 kb.

The conclusion that there is any transcriptional discontinuity at all has to be made with caution, in view of an anomalous result obtained with KC565 (carrying the 7.4 – 10.7 kb segment): even though other mutational cloning results showed that the latter segment was internal to the left-hand *mmy* transcription unit, KC565 transductants gave methylenomycin production zones only marginally smaller than those of the J1507 control. This may be explained (Figure 3) if it is assumed that the cloned fragment in KC565 contains the boundary between two cistrons in a polycistronic *mmy* transcription unit, and that transcriptional readthrough from a vector promoter (probably in the *tsr* region: Figure 2) allows enough transcription of DNA 3′ to the inserted prophage to provide every gene product specified by the operon in quantitites sufficient to give a wild-type phenotype. This result is likely to be encountered most frequently when the site used for cloning in a vector is transcribed from a relatively strong vector promoter, and it may therefore be dependent on the orientation of the insert in the vector and on the particular cloning site used. Examples of this situation have also been found in similar studies of the *S. coelicolor* genes for actinorhodin synthesis (F. Malpartida, personal communication).

The location of mmr. Proof that the *mmr*-like region of pIJ518 contains a functionally intact *mmr* gene was obtained by subcloning the 10.7 – 13.9 kb segment into KC515 to give KC564 (Figure 2). KC564 could transduce methylenomycin resistance into *S. coelicolor* J1501 (ϕC31) which lacked SCP1 and was

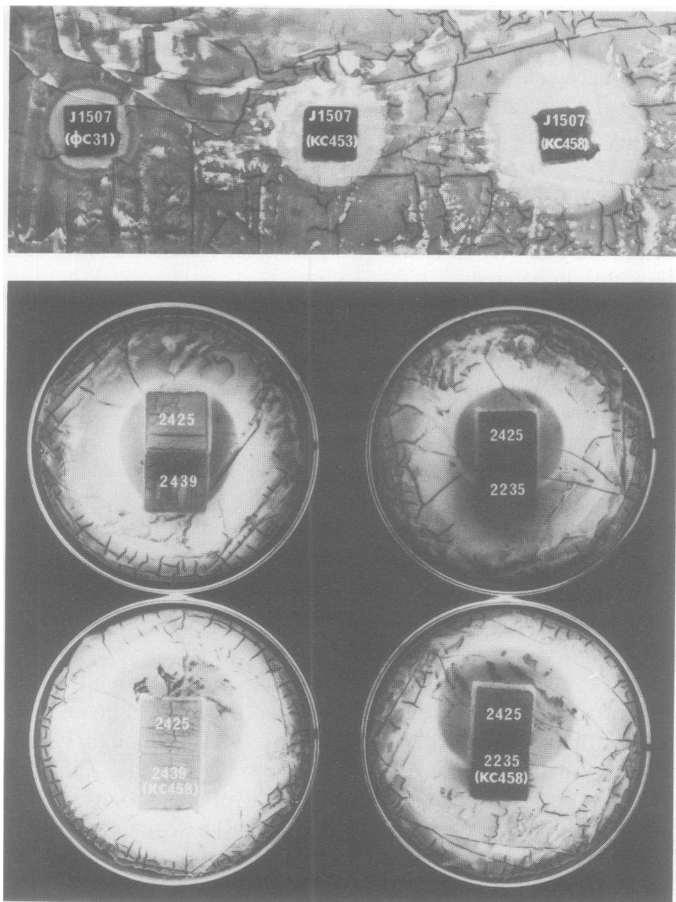

Fig. 4. Overproduction of methylenomycin and its precursors by mutational cloning. **Upper panel.** Relevant J1507 lysogens (see Figure 2) were cultured on solid complete medium (CM: Hopwood, 1967) for 4 days at 30°C, and 1 cm x 1 cm plugs placed on a fresh CM plate spread with spores of the methylenomycin-sensitive (SCP1$^-$) *S. coelicolor* strain J1501 (ϕC31). Zones of inhibition appear light in this panel because of transmitted illumination. **Lower panel.** Strain 2425 is a *mmy* mutant (R39 in Kirby and Hopwood, 1977) able to synthesise methylenomycin from precursors secreted by either of two other *mmy* mutants (2235 and 2439, the SCP1-orange and R11 mutants of Kirby and Hopwood, 1977). The experiments were set up by placing touching 2 cm x 2 cm squares of 2 day CM cultures on CM spread with J1501 (ϕC31). Zones of inhibition appear dark because of surface illumination. In both panels, the plates were photographed after 7 days at 30°C.

therefore Mm^S. (J1501 had first been lysogenised with wild-type ϕC31 to provide a region of homology into which the *attP*-deleted vector could insert by normal recombination: Chater *et al.*, 1982b.) However, Mm^S transductants were obtained with KC567, carrying the 10.5 – 12.5 kb region. Allied with previous evidence, these results suggest that *mmr* is probably at least partially contained within the 0.8 kb separating *Sal*GI site 12.5 from *Pst*I site 13.3 – the very region believed to contain the only *mmy* transcriptional discontinuity discovered in the cloned DNA in pIJ518. Since it has also been found (R. J. Neal and K. F. Chater, unpublished) that a subcloned segment from *Pvu*II site 12.4 to *Pst*I site 13.3 does not confer methylenomycin resistance either, it appears that *mmr* is internal to, and at the right-hand end of, the left-hand *mmy* transcription unit.

The left end of the cloned region of SCP1 contains a regulatory gene. None of the fragments tested from the region left of *Pst*I site 7.4 eliminated methylenomycin production in mutational cloning. Fragments extending or located to the left of the *Pst*I site at 3.1 had no obvious mutagenic effect at all, consistent with their probable origin from a different part of SCP1 (see above); but fragments contained in the segment 3.1 – 4.2 caused striking overproduction [e.g., J1507 (KC458) in Figure 4]. The latter region may lie within a transcription unit involved in negative regulation of methylenomycin biosynthesis (perhaps specifying a repressor protein). All the fragments tested from the adjacent (and overlapping) region from *Pst*I site 4.0 to *Pst*I site 7.4 caused less marked, but nevertheless reproducible, overproduction [e.g., J1507 (KC453) in Figure 4]. It is not clear whether this latter region also encodes regulatory functions, or if the overproduction might result from polar effects of such insertions on expression of the 3.1 – 4.2 kb regulatory region: the overlap between these two regions suggests that they are parts of a single transcription unit. This unit also includes genes for biosynthetic functions, since the overall regulatory region and the leftward *mmy* biosynthetic region both contain *Pst*I site 7.4. (Alternatively, this *Pst*I site could be in a region of overlap between transcription units. This hypothesis would be consistent with the finding that KC563, carrying an insert containing this *Pst*I site, was not overtly mutagenic. However, as shown in Figure 3, fragments internal to transcription units can be non-mutagenic in some situations.)

Assuming that a single operon of at least 9.4 kb is the case, the phenotypes observed in mutational cloning suggest right- to left-wards transcription: with the opposite direction (i.e., with the regulatory gene upstream of production genes), all insertions into this operon should give reduced methylenomycin production.

It was possible that the greatly increased antibiotic activity detected in KC458 and KC459 lysogens was due to a compound other than methylenomycin. This is unlikely, because resistance to the activity was observed in J1501 (ϕC31) further lysogenised by KC412, carrying the 2.5-kb *mmr*-containing *Pst*I fragment. Moreover, overproduction of methylenomycin precursors was observed when KC458 was used to lysogenise strains 2235 and 2439, two *mmy* mutants isolated by Kirby and Hopwood (1977) and shown in that study to secrete precursors. (Detection of precursor production then and in the present study was by co-synthesis with the *mmy* mutant 2425: Figure 4.)

Discussion

The genes for methylenomycin biosynthesis described in this paper are apparently organised into two large transcription units. In the left-hand unit, transcription appears to proceed leftwards for at least 9.5 kb, traversing first *mmr*, then ~5.1 kb of DNA containing sequences essential for biosynthesis (and comprising at least two genes, based on the 'anomalous' mutational cloning result with KC565), then ~3.2 kb inessential for biosynthesis, and possibly fulfilling a regulatory role and, finally, ~1.1 kb presumptively encoding a repressor. The right-hand transcription unit, of at least 6.7 kb, encodes biosynthetic functions. The putative right- to left-ward transcription of the latter region is deduced from an earlier observation that mutational cloning with fragments from the rightmost end (clones A3.13, C8.7, C8.13 and C9.15; Chater and Bruton, 1983) gave Mmy$^-$ transductants devoid of any detectable co-synthetic activity, whereas those obtained using any of three identical fragments (A4.2, B5 and C4.28, consisting of the 13.4 – 16.1 kb interval in Figures 1 and 2) acted as secretors and possibly also as convertors with the tester strain 2425. The former more extensively negative phenotype is consistent with a polar insertion of DNA relatively close to the start site for transcription, and also implies that the

transcription unit is polycistronic with a junction of two cistrons in the A4.2 segment.

This picture of *mmy* and *mmr* transcription leaves open the question of whether there are additional transcripts that overlap the two large ones. For example, an attractive possibility is that transcripts traversing *mmr* could terminate before traversing *mmy* genes during early vegetative growth, allowing resistance to be expressed in advance of production.

The methods and results described here are relevant to the cloning of other antibiotic pathway genes in *Streptomyces*. This study was carried out without the need to use protoplasts of the producing strain, and without the use of conventional mutants. We have estimated (Chater *et al.*, 1985) that shotgun mutational cloning will generate about one non-producing mutant per 1000 transductant colonies screened for antibiotic pathways which, like those for actinorhodin (Malpartida and Hopwood, 1984) and methylenomycin, involve >10 kb of DNA in large transcription units. Secondly, the unambiguous demonstration of intimate linkage of *mmr* to production genes, and evidence of a similar situation for oxytetracycline (Rhodes *et al.*, 1984) and bialaphos (Murakami *et al.*, 1984) resistance and production genes, suggests that shotgun cloning of resistance genes in very large fragments into high capacity SCP2-derived vectors (Lydiate *et al.*, 1985), followed by the analysis of the DNA by mutational cloning, will be an effective route for the future study of production genes for other antibiotics.

Materials and methods

S. lividans 66 (Lomovskaya *et al.*, 1972) (John Innes stock number 1326) was the host for ϕC31 propagation and assay (Chater *et al.*, 1981) and for transfection of protoplasts (Rodicio and Chater, 1982). *S. coelicolor* J1507 (Chater and Bruton, 1983), containing plasmid SCP1 integrated stably into the chromosome in the NF mode (Hopwood *et al.*, 1973), was the usual host for mutational cloning. A ϕC31 lysogen [J1501 (ϕC31)] of an isogenic but SCP1$^-$ *S. coelicolor* strain (J1501; Chater *et al.*, 1982b) was used as host for detecting *mmr* expression and as a Mm^S tester strain to detect methylenomycin production. Transductions (Chater *et al.*, 1982b) were done on solid medium, and transductants selected by replica plating (or sometimes by harvesting and replating) onto plates containing viomycin (20 μg/ml) or thiostrepton (50 μg/ml) to select the *vph* or *tsr* markers of the ϕC31 vectors KC400 (Harris *et al.*, 1983) and KC515 (Rodicio *et al.*, 1985) (see Figure 2). Methylenomycin production and resistance tests were done on solid media as in Chater and Bruton (1983). *E. coli* strain ED8767 (Murray *et al.*, 1977) was the host for cloning with pBR327 (Soberon *et al.*, 1980). *E. coli* procedures were essentially as in Maniatis *et al.* (1982). Viomycin was a gift from E. J. Friend of Pfizer Ltd., Sandwich, Kent, UK and thiostrepton was a gift from S. J. Lucania of E. J. Squibb, New Brunswick, New Jersey, USA.

DNA isolation and in vitro manipulations

DNA was isolated from *Streptomyces* and ϕC31 as in Chater *et al.* (1982a). Plasmid isolation from *E. coli* was as in Birnboim and Doly (1979) or Rodicio and Chater (1982). Techniques and enzymes for the *in vitro* analysis and manipulation of DNA were as in Harris *et al.* (1983). Southern hybridisation (Southern, 1975) was done in conditions of high stringency (Chater *et al.*, 1982b).

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