Location of the genes for anthranilate synthase in *Streptomyces venezuelae* ISP5230: genetic mapping after integration of the cloned genes

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The anthranilate synthase (trpEG) genes in *Streptomyces venezuelae* ISP5230 were located by allowing a segregationally unstable plasmid carrying cloned *S. venezuelae* trpEG DNA and a thiostrepton resistance (tsr) marker to integrate into the chromosome. The integrated tsr was mapped by conjugation and transduction to a location close to tyr-2, between arg-6 and trpA13. A genomic DNA fragment containing trpC from *S. venezuelae* ISP5230 was cloned by complementation of a trpC mutation in *Streptomyces lividans*. Evidence from restriction enzyme analysis of the cloned DNA fragments, from Southern hybridization using the cloned trp DNA as probes, and from cotransduction frequencies, placed trpEG at a distance of 12-45 kb from the trpCBA cluster. The overall arrangement of tryptophan biosynthesis genes in the *S. venezuelae* chromosome differs from that in other bacteria examined so far.

Introduction

The chromosomal organization of genes in the highly conserved tryptophan biosynthesis pathway of bacteria shows remarkable diversity; it varies from an operon-like arrangement in enteric species to a more dispersed pattern of individual genes or clusters of genes in most other groups (Crawford, 1975, 1980, 1989). In streptomycetes, which are soil bacteria belonging to the high-G + C subdivision of Gram-positive eubacteria (Woese, 1987), tryptophan biosynthesis (trp) genes are located on the chromosome in at least two clusters. Conjugational mapping in *Streptomyces coelicolor* A3(2) placed trpD (encoding phosphoribosyl transferase) and trpF (encoding phosphoribosyl isomerase) together at a location distinct from that at which trpA and trpB (encoding the α- and β-subunits, respectively, of tryptophan synthase) and trpC (encoding indolyglycerol phosphate synthase) were positioned (Smithers & Engel, 1974). A similar arrangement was demonstrated in *Streptomyces venezuelae*, where more precise mapping by SV1 phage-mediated transduction established that trpA, trpB and trpC were cotransducible with each other as well as with neighbouring his genes. The group of genes is located within a 45 kb span of DNA (the approximate size of the phage SV1 genome); the gene order hisA–hisB–trpC–trpB–trpA was deduced from relative cotransduction frequencies (Stuttard, 1983; Paradkar et al., 1991b). Also, a 4.2 kb genomic DNA fragment cloned from a third species, *Streptomyces griseus*, complemented trpA, trpB and trpC mutations in *Escherichia coli*, suggesting again that these genes are adjacent on the streptomycete chromosome (Rivero-Lezcano et al., 1990). There is no comparable information on the organization of other tryptophan biosynthesis genes in streptomycetes; in particular, trpE and trpG (encoding the aminase and glutamine amidotransferase activities, respectively, of anthranilate synthase) have not been mapped.

Information about trp gene organization in bacteria can be incomplete because no genetic mapping system is available or, as in *S. venezuelae*, because some classes of mutations are absent from the collection of trp auxotrophs used for genetic analysis. To circumvent these difficulties, trp-containing DNA fragments from bacteria of interest have been cloned by complementation of a relevant trp auxotroph of *E. coli* (Brahamsha & Greenberg, 1987; Sato et al., 1988; Sibold & Henriquet,
1988; Yelton & Peng, 1989). Function and sequence analysis of the cloned DNA and flanking regions has been used to detect the presence of other trp genes. This approach in S. venezuelae identified a 2.4 kb genomic DNA fragment that complemented an E. coli trp auxotroph lacking both TrpE and TrpG functions (Paradkar et al., 1991a). We have now determined the chromosomal location of trpEG by mapping the thiostrepton resistance (tsr) marker of a segregationally unstable trpEG-containing recombinant vector that integrated into the chromosome of S. venezuelae by homologous recombination.

### Methods

**Cultures.** Bacteria and plasmids used are listed in Table 1. Streptomyces were grown on MYM agar (Doull et al., 1985) supplemented with thioacetamide (50 μg ml⁻¹). The E. coli strains were normally grown either on LB medium or on M9 (glucose/ammonium chloride/salts) medium (Sambrook et al., 1989) supplemented with appropriate nutrients; for selecting transformants, ampicillin (100 μg ml⁻¹) was included.

**Transformation procedures.** Protoplasts of S. venezuelae were prepared and transformed with plasmid DNA as described by Sambrook et al. (1989). Plasmid DNA was isolated by the alkaline lysis method of Kieser (Hopwood et al., 1985).

**Hybridization.** Restriction endonuclease digests of genomic DNA were fractionated by electrophoresis in 0.7% agarose gels and were partially depurinated in 0.25 M-HCl for 20 min. The DNA was transferred to a nylon membrane under vacuum and probed with DNA as described by Sambrook et al. (1989). Hybridization was carried out at 65 °C for 14–16 h in a solution containing 20 x SSPE (3 M-NaCl, 0.2 M-sodium phosphate and 0.02 M-disodium EDTA); the membrane was washed at high stringency (0.1 x SSC at 65 °C for 10 min). The 1 x SSC solution contained 0.15 M-NaCl and 0.015 M-sodium citrate, pH 7.0.

**Mapping by transduction.** Approximately 10⁸ spores from a strain carrying presumptive fertility plasmids SVP1 and SVP2 were mixed with excess (10-20-fold) spores from an SVP1- SVP2- strain and spread on nonselective (MYM) medium. After sporulation had occurred, progeny spores were plated on minimal medium supplemented to select one allele from each parent. Approximately 200 of the recombinant colonies that grew were replica-plated to diagnostic media to determine the frequency of each member of each pair of nonselected alleles present. The allele ratio of each new marker positioned it in the gradient of ratios from known markers on either side of the selected alleles; the position chosen was that which minimized the number of recombinants requiring multiple crossovers (Hopwood et al., 1985; Doull et al., 1986).

**Mapping by transduction.** A high-titre lysate of the donor phage SV1 (1 ml) containing 10¹⁰ p.f.u. ml⁻¹ was irradiated at 254 nm to 0.1 % survival. A portion (50 μl) was spread with an equal volume of recipient spores (10⁸ c.f.u. ml⁻¹) on a medium that supported the growth of transductants but not the recipient genotype. Transductants were patched on a medium containing 10 mM-sodium citrate to prevent phage reinfection, and after sporulation were characterized by replica-plating on diagnostic media.

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### Table 1. Bacteria and plasmids used

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<tr>
<th>Strain</th>
<th>Genotype/phenotype</th>
<th>Source/reference</th>
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<tr>
<td>Streptomyces venezuelae</td>
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<tr>
<td>ISP5230</td>
<td>Wild-type</td>
<td>E. Wellington, Warwick University, UK</td>
</tr>
<tr>
<td>AP41</td>
<td>VS194(pDQ189)</td>
<td>This study</td>
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<tr>
<td>AP52</td>
<td>VS194 with modified trpEG</td>
<td>Gene replacement (Paradkar, 1991)</td>
</tr>
<tr>
<td>VS161</td>
<td>tyr-2 thrC1 uraA1</td>
<td>Doull et al. (1986)</td>
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<tr>
<td>VS194</td>
<td>hisA5 adeA10 SVP1<code> SVP2</code></td>
<td>Doull et al. (1986)</td>
</tr>
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<td>VS309</td>
<td>arg-6 thrC1 uraA1 adeA10 strA6</td>
<td>C. Stuttard</td>
</tr>
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<td>VS420</td>
<td>trpA13 pxy-4 cmI-12</td>
<td>Nitrosoguanidine mutagenesis of ISP5230 (Paradkar et al., 1991b)</td>
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<td>trpB14</td>
<td>Nitrosoguanidine mutagenesis of strain TK24 (Paradkar, 1991)</td>
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<tr>
<td>AP2</td>
<td>trpC1</td>
<td>Nitrosoguanidine mutagenesis of strain TK24 (Paradkar, 1991)</td>
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<td>Escherichia coli</td>
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<td>TG1</td>
<td>supE hadA5 thi Δ(lac-proAB) F'(traD36 proABt lacZAM5)</td>
<td>Carter et al. (1985)</td>
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<td>Plasmids</td>
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<td>tsr trpC</td>
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<td>pDQ187</td>
<td>amp trpEG</td>
<td>This study</td>
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<td>pDQ189</td>
<td>tsr amp trpEG (bifunctional)</td>
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<td>pHJL400</td>
<td>tsr, amp, lacZ' (bifunctional)</td>
<td>Larson &amp; Hershberger (1986)</td>
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<td>tsr mel'</td>
<td>Katz et al. (1983)</td>
</tr>
<tr>
<td>ISP5230 Wild-type</td>
<td>tsr, plp, trpC (bifunctional)</td>
<td>Carter et al. (1985)</td>
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Results

Integration of pDQ189 into the S. venezuelae chromosome

The 2.4 kb region of S. venezuelae DNA containing \textit{trpEG} was excised from pDQ187 (Paradkar \textit{et al.}, 1991a) as an \textit{EcoRI–HindIII} fragment and was ligated into the segregationally unstable vector pHJL400 (Larson & Hershberger, 1986) to create pDQ189 (Fig. 1a). To check the stabilities of pDQ189 and pHJL400, each plasmid was isolated from \textit{E. coli} TG1 and used independently to transform \textit{S. venezuelae} VS194 \textit{(hisA6 adeA10 SVP1–SVP2–)} to thiostrepton resistance. When the two types of thiostreton-resistant (Thio\textsuperscript{8}) transformants were propagated on MYM agar without thiostreton selection, VS194(pHJL400) segregated Thio\textsuperscript{8} progeny at an estimated frequency exceeding 98\% of the c.f.u. obtained. In contrast, VS194(pDQ189) under the same conditions lost only 75\% of its initially Thio\textsuperscript{8} resistant population. In the 25\% remaining, the resistant phenotype was lost at only a slow rate during extended propagation on thiostreton-free MYM agar. After a cycle of growth and sporulation under these latter conditions, more than 90\% of the c.f.u. from individual colonies yielded Thio\textsuperscript{8} progeny. Retention of thio-
strepton resistance was attributed to integration of the plasmid into the chromosome by recombination between homologous regions containing trpEG DNA.

Southern analysis with pHLJ400 as a probe showed no hybridization with the genomic DNA of VS194 nor with that of a VS194(pHLJ400) transformant that had lost thiostrepton resistance after nonselective propagation. The genomic DNA of ThioR VS194(pHLJ400) transformants gave the hybridization signal expected for the free plasmid. In contrast, genomic DNA samples of nine independently isolated stable VS194(pDQ189) transformants showed hybridization signals in the region corresponding to high-molecular-mass chromosomal DNA (data not shown). To determine whether pDQ189 had integrated into the chromosome, genomic DNA samples of the parental strain VS194 and a stable VS194(pDQ189) transformant (strain AP41) were digested with restriction enzymes and probed with pDQ189. Since the 2.4 kb trpEG insert but not the vector portion of pDQ189 hybridized with genomic DNA of VS194, and the insert DNA contained no recognition sites for BglII, ClaI or PstI, probing of VS194 genomic DNA digested with these enzymes furnished a restriction map of the chromosomal region containing trpEG. From the sizes of the single hybridizing fragments in digests with BglII (10.5 kb; Fig. 2, lane 4), a mixture of ClaI and PstI (7.8 kb; Fig. 2, lane 6), and a mixture of BglII and PstI (3.2 kb; data not shown), the relationships in Fig. 1(b) were deduced.

The hybridizing fragments in genomic DNA from S. venezuelae strain AP41 differed in number and size from those in VS194 digests, as would be expected if pDQ189 had integrated into the chromosome via single reciprocal crossover(s). Since pDQ189 has no BglII sites, integration of a single copy (8.2 kb) into the homologous region of the 10.5 kb sequence between the two BglII sites in VS194 should have increased the size of this fragment to 18.7 kb. However, the BglII digest of AP41
DNA showed a hybridizing fragment estimated by comparison with λ DNA size markers to be 35–40 kb (see Fig. 2b, lane 3). A DNA fragment of this size would accommodate three or four copies (10.5 + 24.6 or 32.8 = 35.1 or 43.3 kb) of pDQ189 integrated in tandem into the chromosome. In double digests with ClaI and PstI, where VS194 gave a single 7.8 kb hybridizing fragment, AP41 yielded four fragments, estimated to be 3.0, 3.5, 5.2, and 7.3 kb in size (see Fig. 2b, lane 5). Both ClaI and PstI have single sites in the vector region of pDQ189; the 3.0 kb and 5.2 kb fragments thus account for the complete pDQ189 sequence (see Fig. 1a), and their higher signal intensities compared with those of the right and left junction fragments (3.5 kb and 7.3 kb, respectively) are consistent with tandem plasmid integration. Four copies of pDQ189 giving a ratio of four 5.2 kb to three 3.0 kb to one each of the 3.5 kb and 7.3 kb fragments (see Fig. 1c) would be consistent with the Southern blot appearance. Double digests with BglII and PstI gave hybridizing fragments at 2.7, 8.2, and 8.7 kb (data not shown). Their sizes and signal intensities indicate that the 2.7 and 8.7 kb signals were from single-copy junction fragments whereas at 8.2 kb arose from multiple copies of pDQ189 DNA.

Chromosomal location of trpEG

The presence of pDQ189 within the chromosome of AP41 marked the trpEG locus with tsr from the vector. To determine the location of trpEG relative to other chromosomal markers, tsr was mapped by conjugation. Strain AP41 (hisA6 adeA10 tsr SVP1- SVP2-) was mated with various multiply marked SVP1+ SVP2+ strains, and the recombinant progeny were analysed by the single-copy junction fragments whereas that at 8.2 kb arose indicating that the 2.7 and 8.7 kb signals were from single-copy junction fragments whereas at 8.2 kb arose from multiple copies of pDQ189 DNA.

Location of trpEG in relation to trpCBA

Genomic DNA of S. venezuelae was partially digested with MboI and ligated with BglII-digested plJ702 DNA (Katz et al., 1983). When the ligation mixture was used to transform S. lividans AP2 (trpC), a single prototrophic colony was found among 11000 ThioR transformants. The plasmid (pDQ177) isolated from this transformant contained a 9.2 kb insert and efficiently transformed S. lividans AP2 to ThioR prototrophs; in contrast to plJ702, it hybridized at high stringency with S. venezuelae genomic DNA (Fig. 4a, b). Moreover, the number and...
sizes of hybridizing fragments in restriction digests of \textit{S. venezuelae} genomic DNA were consistent with the restriction map of the insert (Fig. 4c), indicating that a continuous piece of chromosomal DNA had been cloned. Detection of only one hybridizing fragment (28 kb) after double digestion with \textit{ClaI} and \textit{PsI} (see Fig. 4b, lane 5) confirmed that the insert had no site for either of these enzymes. The presence of two hybridizing fragments (6.8 and 16 kb) in the \textit{BglII} digest of \textit{S. venezuelae} genomic DNA (see Fig. 4b, lane 4) was consistent with the single site for this enzyme in the restriction map; likewise, the presence of 1.1, 1.2 and 10.5 kb hybridizing fragments in \textit{KpnI} digests (see Fig. 4b, lane 6), and of fragments at 6.2, 4.6 and 3.2 kb in \textit{MluI} digests (see Fig. 4b, lane 2) was compatible with the two restriction sites mapped for each of these enzymes.

Several lines of evidence indicated that \textit{trpC} and \textit{trpEG} are not adjacent. Restriction digests of the cloned DNA containing these genes showed no fragments in common. In addition, Southern hybridization experiments showed that pDQ177 did not hybridize with pDQ181, the recombinant plasmid in which the 6.8 kb \textit{trpEG}-carrying insert was initially cloned. The subeloned 2.4 kb insert containing \textit{trpEG} DNA hybridized to \textit{MluI} digests of \textit{S. venezuelae} genomic DNA to give two fragments (12.0 and 16.5 kb), neither of which was among the hybridizing fragments obtained when \textit{MluI} digests were probed with pDQ177 (containing the 9.2 kb \textit{trpC} DNA). A strain (AP52) derived from VS194 by modifying a unique \textit{MluI} site in the wild-type chromosomal \textit{trpEG} sequence through an allele-exchange procedure gave the same three hybridizing fragments as wild-type \textit{S. venezuelae} when samples of their genomic DNA were digested with \textit{MluI} and probed with pDQ177 (see Fig. 4b, lane 3). Since strain AP52 carried the \textit{trpEG} sequence on a single 28.5 kb \textit{MluI} fragment (unpublished) the result shows that \textit{trpC} does not lie in this segment of DNA, and thus is not within 12 kb on one side or 16.5 kb on the other side of \textit{trpEG}.

**Discussion**

Attempts to clone genes in \textit{S. venezuelae} ISP5230 by complementing host mutations have shown that recombinant plasmids carrying homologous \textit{pab} (Aidoo \textit{et al.}, 1990), \textit{pdx} (Aidoo, 1989) or \textit{trpD} (Paradkar, 1991) DNA integrate readily into the chromosome, and that the \textit{tsr} marker of the integrated vector is cotransducible with neighbouring chromosomal markers. We have exploited this observation to determine the map location of \textit{trpEG} by tagging its chromosomal site with the vector marker and subsequently mapping \textit{tsr} by conjugation and transduction. The procedure is similar to that used to map the location of \textit{IS110} insertion sequences (Chater \textit{et al.}, 1985) and the 2.6 kb minicircle (Lydiate \textit{et al.}, 1989) in \textit{S. coelicolor} A3(2), and also to map the erythromycin resistance gene in \textit{Saccharopolyspora erythraea} (Weber \& Losick, 1988). The approach is particularly useful for chromosomal mapping of those genes for which mutant phenotypes are unavailable. It can be used advantageously in \textit{S. venezuelae} ISP5230, where genetic mapping by transduction is well established (Stuttard, 1989).

Integration of vectors carrying homologous DNA inserts into streptomyctete chromosomes can be achieved with \textit{attP}-deleted \textit{FC}31 phage (Chater \& Bruton, 1983; Buttner \textit{et al.}, 1990), the temperature-sensitive plasmid pGM2 (Muth \textit{et al.}, 1989), nonreplicative \textit{E. coli}-based vectors (Streicher \textit{et al.}, 1989; Kuhstoss \textit{et al.}, 1989; MacNeil \textit{et al.}, 1992), single-stranded vectors (Hilleman \textit{et al.}, 1991) and segregationally unstable plasmids (Larson \& Hershberger, 1990). Among the
latter is pHJL400, a *Streptomyces*-*E. coli* shuttle vector containing the minimum replicon of the *S. coelicolor* plasmid SCP2* but lacking the par region encoding its partition function (Larson & Hershberger, 1986). That this vector might function in *S. venezuelae* ISP5230 was suggested by the earlier use of pDQ101, an SCP2* derivative with a deletion adjacent to the par region, to transform this strain (Aidoo et al., 1990). Whereas pDQ101 was stable in *S. venezuelae*, pHJL400 was lost at a frequency comparable to that observed by Larson & Hershberger (1990) in *S. griseofuscus* transformants grown without thiostrepton selection. Stable trans- 
formants of *S. venezuelae* were readily isolated when the homologous trpEG DNA was present in a recombinant pHJL400 plasmid. Analysis of one such pDQ189 transformant showed 3–4 copies integrated in tandem at the trpEG site. Multiple tandem integration of plasmids carrying the 2.6 kb minicircle has been described in *S. coelicolor* A3(2) (Henderson et al., 1989) and has been observed in *S. griseofuscus* using a vector of the same segregationally unstable type as pHJL400 (C. L. Hershberger, personal communication). Weber et al. (1990) found multiple copies of a recombinant pIJ702 plasmid integrated in the chromosome of *Sac. erythraea*.

The integrated tsr marker (and thus trpEG) was mapped unambiguously between trpA13 and arg-6, close to tyr-2. A location for trpEG near, but not contiguous with, the trpCBA cluster was indicated by cotransduction at low frequency of trpB* and tsr. The possibility that this event was due to two independent phage particles, one carrying tsr and the other carrying trpB*, infecting the same recipient can be excluded because, under optimum conditions, transduction of any given marker occurs at a frequency of only 10^-6 p.f.u.^-1; co-infection by two transducing particles at a frequency of 10^-12 p.f.u.^-1 would be unlikely under the experimental conditions since no more than 10^10 p.f.u. of phage were used for a transduction experiment. Moreover, if spurious cotransduction occurred under these conditions, it should also have been detected with the tyr* allele. For cotransduction of trpB* and tsr from the donor *S. venezuelae* strain AP41 containing tandemly integrated pDQ189, phage SV1 must have packaged a DNA segment with regions capable of homologous recombination at each end of the complementary sequence. Complementarity at the tsr end might not require that the entire tandemly integrated plasmid and neighbouring chromosomal DNA be included in the phage. Since the cloned fragment of homologous DNA present in the plasmid was sufficient for integration, it should also provide the sequence required for a second reciprocal crossover during transduction. To possess the necessary sequence distal to trpB*, the packaged DNA need contain only one full copy of the integrated pDQ189 (to include tsr), and an adjacent additional copy of trpEG for terminus complementarity.

Analysis of the *S. venezuelae* DNA regions carrying trpC and trpEG indicated that they are separated by at least 12 kb. Location of trpE and trpG as a discrete pair separate from trpC, and thus from the trpCBA cluster (Stuttard, 1983; Paradkar et al., 1991a), means that all tryptophan biosynthesis genes except trpF are now mapped in *S. venezuelae*. Auxotrophs carrying the trpF mutation are not yet available in this species. However, trpF has been mapped close to trpD in *S. coelicolor* A3(2), which so far shows a trp gene arrangement similar to that in *S. venezuelae* and *S. griseus* (Smithers & Engel, 1974). Because chromosomal maps of different species of streptomycetes show an overall conservation of linkage relationships (Stuttard, 1988), it is likely that, in most streptomycete species, the trpCBA cluster is at one location and trpEG forms a second locus; trpD and trpF are at a third location, but it is as yet unclear whether they are physically adjacent. In forming three distinct clusters, the streptomycete trp gene arrangement is similar to that in some other bacteria (Sawula & Crawford, 1972; Crawford, 1989; Johnston et al., 1978; Bae et al., 1989); however, the composition of the clusters differs. The trp gene arrangement in *S. venezuelae* is so far unique and contrasts with that in *Brevibacterium lactoferrum*, another member of the high G+C subdivision of Gram-positive bacteria, which has its trp genes in an operon similar to that of enteric bacteria (Matsui et al., 1986).

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