

Identification of a 60 kb region of the chromosome of *Pseudomonas fluorescens* NCIB 10586 required for the biosynthesis of pseudomonic acid (mupirocin)

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Pseudomonic acid (mupirocin) produced by *Pseudomonas fluorescens* is a polyketide antibiotic which blocks isoleucyl-tRNA synthetase. Knowledge of the biosynthetic pathways leading to pseudomonic acid production may help in engineering related antibiotics with other useful properties. To help define these pathways, we have isolated 13 non-producing mutants using Tn5 and Tn1725 mutagenesis. Seven of the Tn5 insertions mapped within a 55 kb region. The remaining insertions, and one gene encoding resistance to pseudomonic acid, mapped at separate locations. DNA that overlapped the seven clustered Tn5 insertions was isolated on a series of clones, extending over a region in excess of 60 kb. Non-producing mutants generated via gene disruption suggest that the biosynthetic cluster extends throughout this region.

Keywords: *Pseudomonas fluorescens*, mupirocin, pseudomonic acid, polyketide antibiotic, gene cloning

INTRODUCTION

Pseudomonic acid A (mupirocin) is an antibiotic produced by the Gram-negative, aerobic bacterium *Pseudomonas fluorescens*. It inhibits isoleucyl-tRNA synthetase, by blocking the formation of the enzyme. Ile-AMP complex, thus preventing the incorporation of isoleucine into growing peptide chains (Hughes & Mellows, 1978, 1980). Its primary use has been in the treatment of skin infections by members of the bacterial genus *Staphylococcus* (e.g. Scully *et al.*, 1992; Rice *et al.*, 1992), but it has also been used to treat cutaneous candidiasis (Rode *et al.*, 1991) and in the control of burn wound infections (Rode *et al.*, 1988).

Pseudomonic acid consists of an unsaturated pentaketide and saturated C₉ fatty acid, 9-hydroxynonanoic acid, esterified by a branched C₅ unit (Fuller *et al.*, 1971; Chain & Mellows, 1974, 1977; Alexander *et al.*, 1978; Fig. 1). The pentaketide and C₅ unit together comprise monic acid, a complex molecule containing epoxide, diol and

tetrahydropyran functions. In addition to pseudomonic acid A, fermentations yield other minor forms of pseudomonic acid that comprise around 5% of the total product. These include pseudomonic acid B, which possesses an additional hydroxyl group in the ring (Chain & Mellows, 1977), pseudomonic acid C, in which a double carbon-carbon bond replaces the epoxide group (Clayton *et al.*, 1980, 1982), and pseudomonic acid D, which possesses an unsaturated fatty acid side chain (O'Hanlon *et al.*, 1983). The contribution that these forms of pseudomonic acid make to the overall antibiotic activity is unclear.

The biosynthetic pathway to pseudomonic acid has not been characterized completely. However, the C₁₂, C₅ and C₉ units have been identified as products of separate

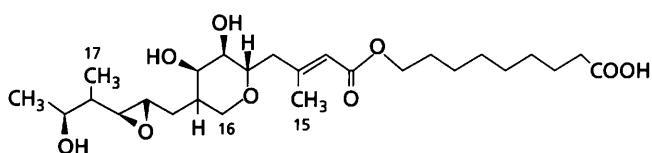


Fig. 1. Structure of pseudomonic acid A (mupirocin). The carbons at positions 15, 16 and 17 are indicated.

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pathways that are subsequently assembled together (Martin & Simpson, 1989). An early suggestion that the C₅ unit and 9-hydroxynonanoic acid are derived from hydroxymethylglutaryl-CoA (HMG-CoA; Feline *et al.*, 1977) has been disproved (Martin & Simpson, 1989). Instead, the entire molecule is derived from acetate, with the exception of C-16 and C-17, which are derived from the methyl of methionine (Feline *et al.*, 1977). It is believed that the acetate units are incorporated into monic acid and 9-hydroxynonanoic acid via a classical polyketide mechanism.

Many genes encoding components of polyketide synthases have been cloned and characterized (see Hopwood & Sherman, 1990, for a review), revealing strong conservations across a wide range of species. The genes may be organized generally in one of two ways. For multifunctional type I synthases, all the component activities are encoded by large ORFs (e.g. Cortes *et al.*, 1990; Donadio *et al.*, 1991; MacNeil *et al.*, 1992). Moreover, several multifunctional enzymes may be involved in specific cycles of the same biosynthetic pathway, such that several large ORFs can be clustered together extending across a region in excess of 60 kb (e.g. Donadio *et al.*, 1991; MacNeil *et al.*, 1992). Such organizations appear to have evolved as a means to facilitate the complex programming required for some polyketide biosyntheses (Donadio & Katz, 1992). For type II synthases, the individual proteins are encoded by separate genes (e.g. Sherman *et al.*, 1989; Bibb *et al.*, 1989; Bergh & Uhlen, 1992), although there is evidence for genes encoding proteins with more than one activity (e.g. Sherman *et al.*, 1991; Summers *et al.*, 1992). At least in *Streptomyces*, the corresponding genes involved in the biosynthesis of different polyketides are organized very similarly (Hopwood & Sherman, 1990).

Although members of the genus *Pseudomonas* produce a number of polyketide-derived compounds (e.g. Fuller *et al.*, 1971; Nishiyama *et al.*, 1976; Mitchell & Young, 1978; Bender *et al.*, 1993; Cuppels *et al.*, 1986; Defago, 1993; Ullrich *et al.*, 1994), few studies have been undertaken to characterize the biosynthetic genes. Recently, a characterization of a 43 kb region involved in the biosynthesis of the polyketide phytotoxin, coronatine, by *Pseudomonas syringae* was described (Young *et al.*, 1992; Ullrich *et al.*, 1994). This paper describes the identification of mutations blocking pseudomonic acid production and the location of a cluster of biosynthetic genes.

METHODS

Bacterial strains and culture conditions. The pseudomonic acid producers *P. fluorescens* NCIB 10586 and its reisolate *P. fluorescens* 332 were used as parental strains. *Escherichia coli* HB101 (Boyer & Roulland-Dussoix, 1969) was used as the donor in conjugal matings, *E. coli* C600 (B. Bachmann, Yale University) and *E. coli* JM83 (Yanisch-Perron *et al.*, 1985) were used for routine cloning experiments, and *E. coli* P2392 (Raleigh *et al.*, 1989) was used for propagation of the λ EMBL3 library. *Bacillus subtilis* 1604 (Moir *et al.*, 1979) was used as the sensitive strain in bioassays. *P. fluorescens* strains were grown at 30 °C and *E. coli* and *B. subtilis* strains were grown at 37 °C, routinely in

L-broth or on L-agar (Kahn *et al.*, 1979). Where appropriate, chloramphenicol was used at 800 $\mu\text{g ml}^{-1}$ for *P. fluorescens*, kanamycin at 50 $\mu\text{g ml}^{-1}$ for both *P. fluorescens* and *E. coli*, and benzylpenicillin at 200 $\mu\text{g ml}^{-1}$ for *E. coli*.

Plasmids and phage. pUC18 (Yanisch-Perron *et al.*, 1985) and pAT153 (Twigg & Sherratt, 1980) were used as general cloning vectors in *E. coli*. pRK2013 (Figurski & Helinski, 1979) was used to mobilize plasmids from *E. coli* into *P. fluorescens*. pLG221 (Boulnois *et al.*, 1985) and pSUP301::Tn1725 (Simon *et al.*, 1983; Tn1725 was introduced by V. Shingler, University of Umeå, Sweden) were used to generate Tn5 (Berg, 1977; Berg *et al.*, 1975) and Tn1725 (Ubben & Schmitt, 1986) mutants in *P. fluorescens*. λ EMBL3 (Frischauf *et al.*, 1983) was used to prepare a genomic library of *P. fluorescens*.

Molecular biological techniques. Plasmid DNA isolations and transformations of *E. coli* and *P. fluorescens* strains were performed according to standard procedures (Sambrook *et al.*, 1989). Restriction enzymes, DNA ligase, calf intestinal alkaline phosphatase (CIP) and DNA polymerases were purchased from Gibco BRL, New England Biolabs or Northumbria Biologicals and used according to the manufacturer's instructions.

Genomic library construction and screening. Two genomic libraries of *P. fluorescens* NCIB 10586 were constructed, in the plasmid pAT153 (Twigg & Sherratt, 1980) and in λ EMBL3 (Frischauf *et al.*, 1983). The pAT153 library was made by subcloning total *Clal*-digested DNA into *Clal*-linearized CIP-treated pAT153. The library was maintained as a collection of 8000 separate transformants and as a total plasmid preparation. The λ EMBL3 library was constructed by subcloning *Sau3AI*-digested DNA (size-fractionated on a sucrose gradient to enrich for fragments in the range 14–25 kb) between *Bam*HI digested, CIP-treated left and right arms of the phage vector. This was maintained at 4 °C as a suspension of approximately 6000 p.f.u. ml⁻¹ in SM (0.05 M Tris/HCl, pH 7.5, 0.1 M NaCl, 8 mM MgSO₄·7H₂O, 0.004% gelatin) containing a drop of chloroform, and propagated on *E. coli* P2392 according to standard procedures (Sambrook *et al.*, 1989). The two libraries were screened by colony or plaque hybridization according to standard procedures (Sambrook *et al.*, 1989).

Transposon mutagenesis. Tn5 and Tn1725 mutants of *P. fluorescens* were isolated using the suicide vectors pLG221 and pSUP301::Tn1725 respectively. Both plasmids can be conjugally transferred into *P. fluorescens*, but cannot replicate in this host. As a consequence, *P. fluorescens* transconjugants that acquired either kanamycin resistance or chloramphenicol resistance arose as a result of transposition of Tn5 or Tn1725 into the chromosome.

Conjugal matings. Plasmids were mobilized into *P. fluorescens* via the helper plasmid pRK2013, which encodes all the functions necessary for conjugal transfer of the broad-host-range plasmid RK2 (Figurski & Helinski, 1979) but cannot self-propagate in *P. fluorescens* because it is based on the ColE1 replicon. *E. coli* donor cells containing pRK2013 and the plasmid to be mobilized were grown to late exponential phase and *P. fluorescens* recipient cells were grown to either mid or late exponential phase, both in 5 ml L-broth supplemented with appropriate antibiotics. Then 0.5 ml of each culture was filtered on to a sterile 0.45 μm filter and incubated on a dry L-agar plate overnight at room temperature or at 30 °C. The mating mixture was resuspended in 1 ml sterile saline solution and 0.1 ml aliquots of appropriate dilutions were spread on plates containing the appropriate antibiotic to select for transfer of the plasmid and pseudomonic acid (at 750 $\mu\text{g ml}^{-1}$) to kill the *E. coli* donors. For the isolation of Tn5 mutants and gene disruption

mutants, kanamycin was used at a concentration of 50 µg ml⁻¹, and for the isolation of Tn1725 mutants, chloramphenicol was used at a concentration of 800 µg ml⁻¹. In general, trans-conjugants arose after 2–4 d incubation at 30 °C and were then subcultured on to fresh plates.

Analysis of *P. fluorescens* chromosomal DNA using pulsed field gel electrophoresis (PFGE). Preparation of *P. fluorescens* chromosomal DNA, subsequent endonuclease digestion and separation of restriction fragments using PFGE has been described previously (Whatling & Thomas, 1993).

Southern hybridizations. These were performed in general as described by Sambrook *et al.* (1989). DNA probes were labelled according to a random priming technique, using [α -³²P]dCTP (10 µCi µl⁻¹; 370 kBq µl⁻¹), random hexanucleotides (Sigma; at 90 absorbance units ml⁻¹) and Klenow DNA polymerase (Gibco BRL). Pre-hybridization and hybridization buffers contained 5 × SSC, 50% formamide, 5 × Denhardt's reagent (0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% BSA), 50 mM sodium phosphate, pH 6.5, 0.1% SDS and 250 µg denatured salmon sperm DNA ml⁻¹, and were used at 42 °C. Washes were performed twice using 2 × SSC, 0.1% SDS and 0.1 × SSC, 0.1% SDS for 30 min at 45 °C.

Gene disruption strategy. pUC18, which cannot replicate in *P. fluorescens*, was used to construct a vector for performing gene disruption analysis in *P. fluorescens*, since only if it carries chromosomal DNA derived from *P. fluorescens* can it propagate following homologous recombination with the chromosome. Two features were incorporated into pUC18 to render it appropriate for performing gene disruption. First, the *aph* gene from Tn5 was introduced on a 1.4 kb *SalI*–*HindIII* fragment to give pCAW5, allowing selection for recombinants using kanamycin (*P. fluorescens* NCIB 10586 was resistant to 200 µg penicillin ml⁻¹). Second, the origin of transfer (*oriT*) from RK2, which allows mobilization of plasmids via the helper plasmid pRK2013 from *E. coli* to *P. fluorescens*, was introduced on a 0.7 kb *BamHI* fragment, giving pCAW5.1. pCAW5.1 was linearized by partial digestion with *BamHI*, end-filled with Klenow, and re-ligated to yield pCAW5.2 (Fig. 5) which contains a single *BamHI* site to the left of *oriT*. In general, between 10 and 100 transconjugants were obtained per ml of mating mixture, the frequency decreasing with the size of the *Sau3AI* insert.

Bioassay for pseudomonic acid. *P. fluorescens* colonies were picked on to Mupirocin Production Agar plates (MPA; containing, per litre: 2.3 g yeast extract, 1.1 g glucose, 2.6 g Na₂HPO₄, 2.4 g KH₂PO₄, 5.0 g (NH₄)₂SO₄, 1.5% Oxoid agar) and grown for 1–2 d at 30 °C. Plates were overlaid with nutrient agar containing 4 ml of a culture of *B. subtilis* 1604 grown to late exponential phase and 0.5 ml of 5% (w/v) 2,3,5-triphenyl-tetrazolium chloride (TTC) per 100 ml. After incubation overnight at 37 °C antibiotic production was recorded as clear zones around the bacterial colonies, against a red background (resulting from the reduction of TTC). Positive and negative controls were included on each plate. Levels of antibiotic production were measured in terms of the diameter of the zone of inhibition: parental levels gave zones of inhibition 3–4 cm in diameter; intermediate-level production was scored when the zones of inhibition were less than half this diameter. To measure antibiotic levels in culture supernatants from shake-flask fermentations, samples were subjected to an overnight hole-in-plate bioassay at 32 °C using Difco Sensitivity Test agar seeded with Difco *B. subtilis* spore suspension (0.8 ml per 300 ml molten agar). Zones of antibiosis produced by known concentrations of antibiotic were determined to estimate the titres of pseudomonic acid present.

HPLC analysis of *mup* non-producers. Extracts of wild-type and *mup* non-producers were analysed on a Waters C18 column using a buffer made from stock solutions of 0.5 M acetic acid (pH 6), 75% (v/v) methanol and deionized H₂O + 1% methanol, which were mixed in the ratio of 7:80:13. The column was run with a flow rate of 2.5 ml min⁻¹, at 40 °C, and a sample size of 10 ml was injected. The retention time for pseudomonic acid A detected at a standard wavelength of 260 nm was approximately 2.7 min.

RESULTS

Identification of *mup* mutants defective in pseudomonic acid biosynthesis

Several thousand *P. fluorescens* 332 Tn5 and *P. fluorescens* NCIB 10586 Tn1725 mutants were individually bio-assayed for pseudomonic acid production, first with plate bioassays, and then by testing culture filtrates from scaled-down shake-flask fermentations. Ten Tn5 and three Tn1725 stable non-producers were identified. HPLC analysis of culture extracts showed that in each case peaks corresponding to pseudomonic acid A or B were not present, confirming that the mutants do not produce pseudomonic acid (data not shown). No intermediate compounds related to pseudomonic acid biosynthesis could be detected in the mutants, and the mutants appeared to have normal morphology, exhibited no imbalance in metabolism, grew with normal generation times and showed no auxotrophic defects.

The Tn5 and Tn1725 insertions were mapped to various *SalI* and *SmaI* fragments, respectively, by probing chromosomal DNA digested with each enzyme with radioactively labelled transposon DNA. Most mutants contained a single transposon insertion. However, the Tn1725 mutant 22 and Tn5 mutant 90 were shown to contain two separate insertions; in these two mutants the actual insertion responsible for the non-producing phenotype has not been elucidated.

A cluster of Tn5 insertions in mutants blocked for pseudomonic acid production

Tn5 and the surrounding chromosomal DNA was sub-cloned into pAT153 from the genome of *mup* mutants either as *EcoRI* fragments or as *ClaI* fragments. The plasmid containing Tn5 from mutant 10, pBROC130, contained a 9.4 kb *EcoRI* fragment with Tn5 located approximately 3 kb from one end. To obtain a clone lacking Tn5, labelled chromosomal DNA from pBROC130 was used to probe a library of *ClaI* fragments in pAT153. A plasmid containing an 11.8 kb fragment was identified. This plasmid, pBROC131, contained 3.3 kb of DNA in common with pBROC130 such that together they contained chromosomal DNA extending over a 17.6 kb region (Fig. 2a).

To determine whether other transposon insertions mapped within the region covered by pBROC130 and pBROC131, the *ClaI* fragment from pBROC131 was used to probe *EcoRI*-digested DNA from the Tn5 mutants and *SmaI*-digested DNA from the Tn1725 mutants. In the

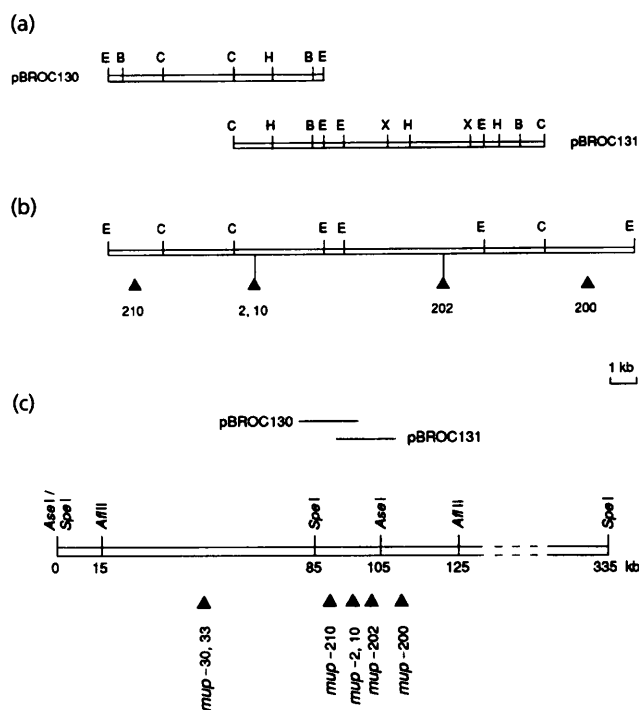


Fig. 2. Map of the chromosomal region studied in this paper. (a) Overlapping restriction maps of the chromosomal DNA present in pBROC130 and pBROC131. The DNA in pBROC130 was split by Tn5 inserted at the position for mutation 10. (b) Combined map of the chromosomal region carrying the cluster of mutations *mup*-2, -10, -200, -202 and -210. Abbreviations: E, *EcoRI*; B, *Bam*HI; C, *Cl*aI; H, *Hind*III; X, *Xho*I. (c) Partial *Afl*II, *Ase*I and *Spe*I restriction map across the region of the *P. fluorescens* chromosome containing *mup*-30, -33, -210, -2, -10, -202 and -200. The order of the left-hand *Ase*I and *Spe*I sites, which appear to map close, has not been determined.

case of the Tn5 mutants 2, 200, 202 and 210, one of the *EcoRI* fragments that hybridized was approximately 5.6 kb larger than the parental band, indicating that Tn5 in these mutants mapped within *EcoRI* fragments at least partly overlapping the 11.8 kb *Cl*aI fragment. By constructing an *EcoRI* map for this region, the approximate

location of each Tn5 insertion was ascertained (Fig. 2b). A similar mapping experiment using DNA adjacent to Tn5 from mutant 33 indicated that Tn5 in mutant 30 mapped close to this insertion (possibly within the same 3.2 kb *Sal*I fragment), but did not reveal linkage with any of the other mutants.

At least four separate regions of the *P. fluorescens* chromosome could be involved in the biosynthesis of pseudomonic acid

To ascertain the overall distribution of the Tn5 insertions in the *P. fluorescens* chromosome, their locations were mapped to *Afl*II, *Ase*I and *Spe*I fragments. These enzymes cleave the *P. fluorescens* NCIB 10586 chromosome into between 40 and 60 fragments, in the size range from 5 to 300 kb. This relatively low frequency of site recognition reflects the GC content of *P. fluorescens* DNA (approximately 61 mol%; Palleroni, 1975) and the AT-richness of the target sites (*Afl*II, C/TTAAG; *Ase*I, AT/TAAT; *Spe*I, CA/CTAGT). None of the three enzymes cleaves within Tn5. When labelled Tn5 was used to probe *Ase*I-, *Afl*II- and *Spe*I-digested chromosomal DNA from several Tn5 mutants a single strongly hybridizing band was observed for each Tn5 insertion. The data in Table 1 clearly indicate that the Tn5 mutations *mup*-75, -90, -113 and -202 mapped to different *Afl*II, *Ase*I and *Spe*I fragments, suggesting that genes located in at least four regions of the *P. fluorescens* chromosome are involved in some way with pseudomonic acid biosynthesis.

The Tn5 mutations *mup*-30, -33, -202 and -210 map within a 90 kb *Afl*II-*Ase*I fragment

The mutations *mup*-30, -33, -202 and -210 mapped in a 110 kb *Afl*II fragment and a 105 kb *Ase*I fragment (Table 1). This raised the possibility that *mup*-30 and *mup*-33 could map within the vicinity of the biosynthetic cluster disrupted by *mup*-202 and *mup*-210. However, whereas *mup*-202 and *mup*-210 mapped within a 250 kb *Spe*I fragment, *mup*-30 and *mup*-33 mapped within a 90 kb *Spe*I fragment. To determine whether it was the same 110 kb

Table 1. Approximate sizes (kb) of the *Afl*II, *Ase*I and *Spe*I fragments which hybridize strongly with Tn5 probe in seven non-producing mutants

Tn5 mutant	<i>Afl</i> II	<i>Ase</i> I	<i>Spe</i> I	<i>Afl</i> II/ <i>Ase</i> I	<i>Afl</i> II/ <i>Spe</i> I	<i>Ase</i> I/ <i>Spe</i> I
<i>mup</i> -30	110	105	85	90	70	85
<i>mup</i> -33	110	105	85	90	70	85
<i>mup</i> -75	40	55	300	ND	ND	ND
<i>mup</i> -90	15, 35	25, 40	30, 150	ND	ND	ND
<i>mup</i> -113	25	20	90	ND	ND	ND
<i>mup</i> -202	110	105	250	90	40	20
<i>mup</i> -210	110	105	250	90	40	20

ND, Not determined.

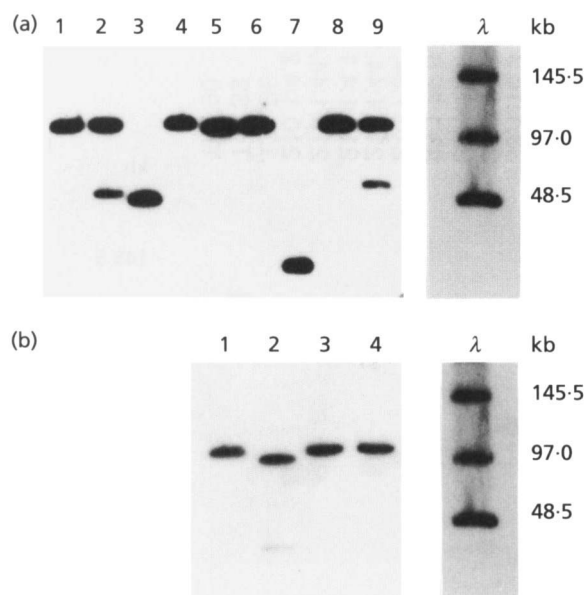


Fig. 3. Demonstration that the Tn5 insertions in the non-producing mutants *mup*-30, -33, -202 and -210 are located within the same *AffII* and *AseI* fragment. (a) Lanes 1–4, *AffII*-digested DNA from mutants 30, 202, 90 and 33, respectively, probed with Tn5. Lanes 5–9, *AseI*-digested DNA from mutants 30, 33, 113, 202 and 210, respectively, probed with Tn5. λ , lambda DNA concatemers. (b) Lanes 1–4, *AseI*-digested DNA from mutants 30, 113, 33 and 202 probed with a 3.0 kb *XhoI* fragment from pBROC131. λ , lambda DNA concatemers. The hybridizing fragment for mutants 30, 33 and 202 is retarded relative to that for mutant 113 due to the presence of Tn5. Electrophoresis conditions: 1.3% agarose gel, voltage gradient 5.3 V cm⁻¹, switch interval ramped from 10 to 35 s over a period of 36 h.

AffII and 105 kb *AseI* fragments which contained both groups of mutations, DNA from mutants *mup*-202, -30, -33 and -75 digested with each enzyme was probed with chromosomal DNA located between *mup*-202 and *mup*-210. In both cases, the hybridizing band for mutants *mup*-202, -30 and -33 was retarded compared to that for mutant 75 (Fig. 3a, b), indicating that they contained Tn5 in these mutants and therefore that *mup*-202, *mup*-30 and *mup*-33 mapped to the same *AffII* and *AseI* fragments.

The *AffII*–*AseI*, *AffII*–*SpeI* and *AseI*–*SpeI* fragments in which *mup*-30, -33, -202 and -210 mapped were identified by probing double digests with Tn5 (Table 1). Alignment of the fragments produced by single and double digestion in a restriction map implied that the 85 kb and 250 kb *SpeI* fragments were adjacent (Fig. 2c), a fact that was confirmed following the identification of a *SpeI* site in pBROC130 close to *mup*-210: when a 2.7 kb *EcoRI*–*ClaI* fragment traversing this *SpeI* site was used to probe *SpeI*-digested DNA, both the 85 kb and 250 kb fragments hybridized. It was also possible to locate one end of the 105 kb *AseI* fragment at a site 1.5 kb from the right-hand end of the 11.8 kb *ClaI* fragment in pBROC131. When a 1.4 kb *BamHI*–*EcoRI* fragment overlapping this site was

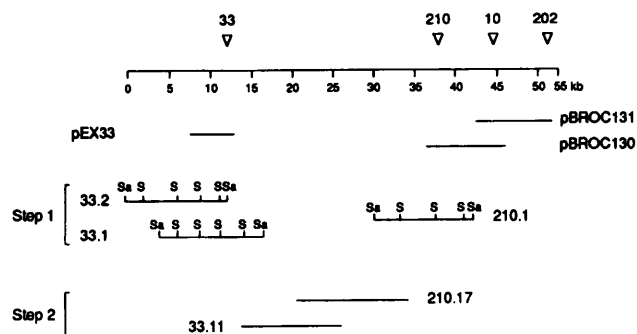


Fig. 4. Chromosome walking strategy used to isolate the DNA located between *mup*-33 and *mup*-210 in the *P. fluorescens* genome. In the first step, a 0.7 kb *EcoRI*–*BglII* fragment from pBROC130 and a 0.7 kb *KpnI* fragment from pEX33 (a derivative of pAT153 containing Tn5 rescued from mutant 33 on a 6.9 kb *ClaI* fragment) were used to probe a λ EMBL3 genomic library. Of several clones identified, λ CAW210.1 extended the furthest from *mup*-210, and λ CAW33.1 and λ CAW33.2 extended furthest from *mup*-33. *SaI* restriction maps were constructed for each of these phages, and these are presented since they were used in gene disruption experiments (Fig. 6). Neither λ CAW33.1 nor λ CAW33.2 shared any overlap with λ CAW210.1. In the second step, the 1.8 kb *SaI* end fragment from λ CAW33.1 and 3.4 kb *SaI* end fragment from λ CAW210.1 were used to probe the λ EMBL3 library. The two clones that extended furthest from these probes were λ CAW33.11 and λ CAW210.17, respectively. Restriction mapping/probing indicated that these phages possessed approximately 6 kb of DNA in common. Abbreviations: Sa, *Sau3AI*, S, *SaI*.

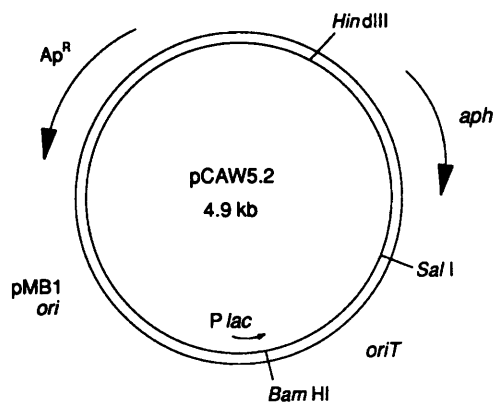


Fig. 5. Map of pCAW5.2, a derivative of pUC18 used to perform gene disruption analysis in *P. fluorescens*. Symbols: *aph*, aminoglycoside phosphotransferase gene from Tn5; *oriT*, origin of transfer from RK2; *Ap^R*, ampicillin-resistance gene of pUC18; *Plac*, promoter of *lacZ* gene. Three *AseI* sites are located in the pUC18 portion located at positions 576, 635 and 1870 relative to the standard pUC18 sequence, i.e. 142, 201 and 1436 bp clockwise from the *BamHI* site shown on the map.

used to probe *AseI*-digested DNA, the 105 kb fragment and a 25 kb fragment hybridized. The Tn5 insertion in mutant 200 probably mapped within this 25 kb fragment.

The smallest fragment that overlapped *mup*-30, -33, -202 and -210 was a 90 kb *AffII*–*AseI* fragment. *mup*-202 and

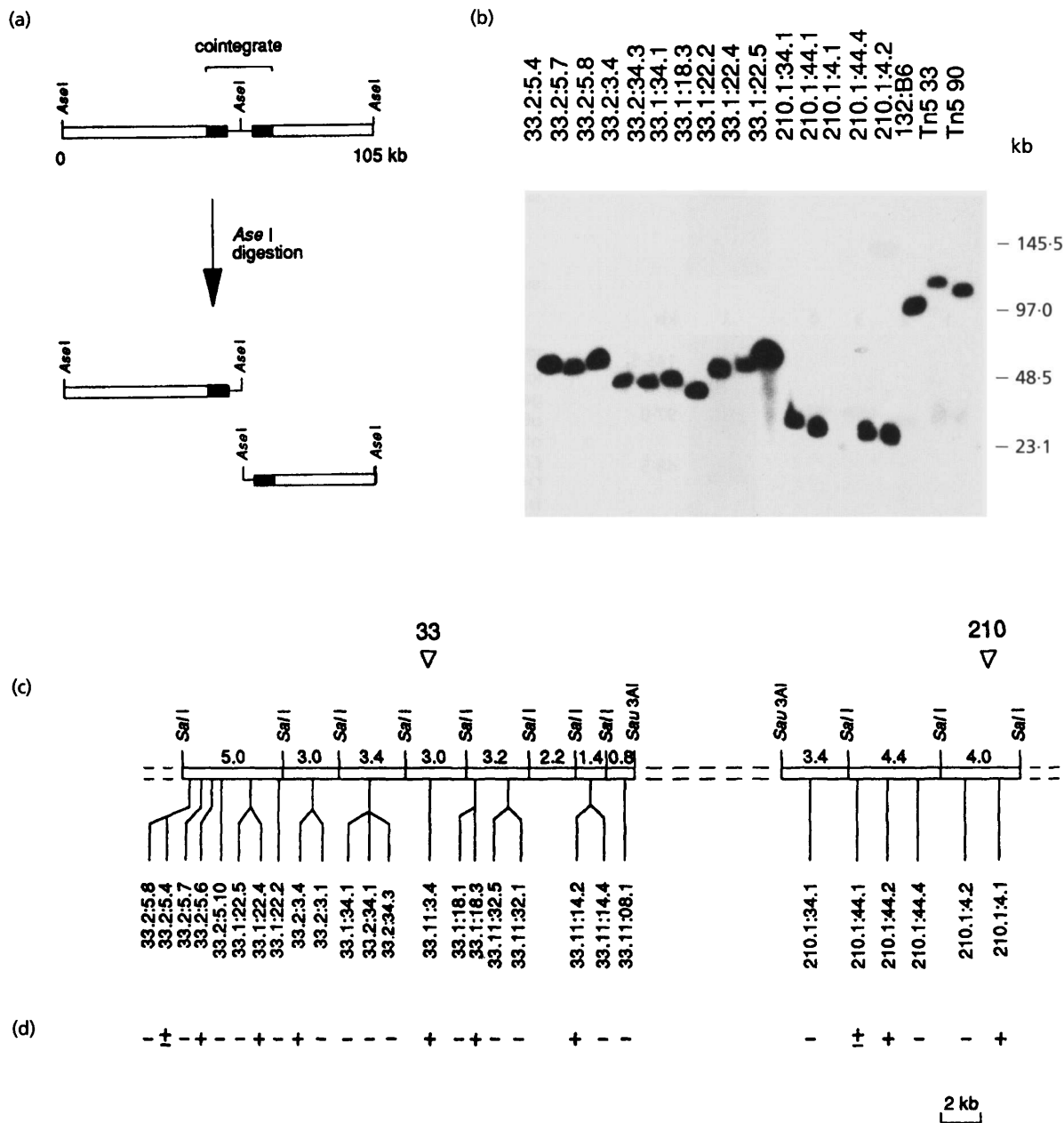


Fig. 6. Effect on pseudomonic acid production of integrated pCAW5.2 derivatives in a 40 kb region of the *P. fluorescens* chromosome extending across *mup-33* and up to *mup-210*. (a) Mapping the sites of cointegration of pCAW5.2 derivatives. Cointegration of pCAW5.2 introduces three additional closely spaced *AseI* sites (shown as a single site in the vector) into the chromosome. Consequently, when chromosomal DNA from a recombinant is digested with *AseI* (identified by an appropriate probe), the parental *AseI* fragment containing pCAW5.2 is separated into two fragments. (b) *AseI*-digested DNA from a range of *P. fluorescens* NCIB 10586 recombinants containing cointegrated pCAW5.2 and the Tn5 mutants *mup-33* and -90 probed with the 3.0 kb *XhoI* fragment from pBROC131. For mutant 90, the probe hybridized to the parental *AseI* fragment. In the case of mutant 33, this same fragment was retarded due to the presence of Tn5. For the recombinants, the 105 kb fragment was separated into two fragments, only one of which hybridized to the probe (i.e. the right-hand fragment shown in a). The low intensity of the signal for *P. fluorescens*::210.1:4.1 resulted from a low yield of DNA from this strain. (c) Sites of integration of pCAW5.2 derivatives in the *P. fluorescens* chromosome. Each derivative of pCAW5.2 was classified according to the origin of the *Sau3AI* fragment used to direct recombination. For example, constructs that contained a *Sau3AI* fragment isolated from the 5 kb *SalI* fragment from λ CAW33.2 were designated 33.2:5.N, where N was determined by the number of constructs derived from the same *SalI* fragment. The numbers above the map correspond to the size of the *SalI* fragment in which each pCAW5.2 derivative integrated. In cases where it was not possible to resolve the order of insertions within a single *SalI* fragment, the corresponding constructs have been grouped together. There is a gap in the map corresponding to DNA uniquely present in λ CAW210.17. Gene disruption analysis has not been performed using DNA isolated from this phage. (d) Phenotypes of recombinants, as determined by bioassay. +, recombinants producing parental level of antibiotic as estimated from zones of inhibition around colonies; ±, recombinants producing intermediate level of antibiotic; -, recombinants producing undetectable level of antibiotic.

mup-210 mapped approximately 5 kb and 15 kb from the *Ase*I site, respectively. Consequently it was possible that *mup*-30 and *mup*-33 could map anywhere up to 75 kb from *mup*-210. To determine the actual distance, the location of *mup*-30 and *mup*-33 was mapped more accurately.

***mup*-33 and *mup*-210 map approximately 24 kb apart**

To determine the distance separating *mup*-33 and *mup*-210 the intervening DNA was isolated on a series of overlapping clones from a genomic library of *P. fluorescens* NCIB 10586 in λ EMBL3. This was achieved by walking from both mutations to a common region (Fig. 4). Two walking steps were required to bridge the gap, providing genomic clones that extended over a total of 44 kb. Restriction analysis of the DNA located between *mup*-33 and *mup*-210 indicated that the two mutations are approximately 24 kb apart (this represents a maximum estimate: λ CAW33.11 and λ CAW210.17 were shown to contain at least three fragments in common, of sizes 2.5, 1.4 and 0.7 kb, but the exact limits of the overlap are likely to occur within different fragments). The Tn5 insertions in this region of the chromosome therefore extend over a total of 55 kb (i.e. from *mup*-33 to *mup*-200). In addition to the 24 kb of DNA between *mup*-33 and *mup*-210, a further 13 kb was isolated that extended in the opposite direction from *mup*-33 (i.e. in λ CAW33.2), and approximately 6 kb of DNA was isolated that extended beyond *mup*-200 (these latter clones have not been subject to detailed analysis). Consequently, over 60 kb DNA from this region is available in cloned form.

Genes involved in the biosynthesis of pseudomonic acid may be located throughout the 24 kb region between *mup*-33 and *mup*-210, and for at least 13 kb beyond *mup*-33

A gene-disruption strategy was used to determine whether the DNA located between *mup*-33 and *mup*-210 contained genes involved in pseudomonic acid biosynthesis. It was reasoned that if biosynthetic genes were present, disruption could block the production of pseudomonic acid A. This type of approach has been used successfully to map other antibiotic biosynthetic genes (Chater & Bruton, 1983; Malpartida & Hopwood, 1986; Weber *et al.*, 1990).

Gene disruption employed pCAW5.2 (Fig. 5), and relied on the failure of this plasmid to replicate autonomously in *P. fluorescens* (see Methods). So that DNA around and between *mup*-33 and *mup*-210 could be targeted, DNA fragments from λ CAW33.2, λ CAW33.1, λ CAW33.11 and λ CAW210.1 were subcloned into pCAW5.2. This was done by isolating individual *Sa*I fragments, digesting with *Sau*3AI and subcloning the resulting fragments into the single *Bam*HI site of pCAW5.2. Derivatives were obtained for each *Sa*I fragment, containing *Sau*3AI fragments ranging from 0.1 to 1 kb in size.

Individual pCAW5.2 derivatives were conjugally transferred into *P. fluorescens* NCIB 10586 and transconjugants

that acquired the kanamycin-resistance phenotype of pCAW5.2 were identified for further analysis. To confirm that pCAW5.2 had integrated, chromosomal DNA was isolated from representative transconjugants and digested with *Ase*I (Fig. 6). This also allowed the approximate location of each integrate to be determined. Recombinants that were shown to contain homologously integrated pCAW5.2 were subsequently bioassayed for pseudomonic acid A production (Fig. 6).

Recombinants separated into three phenotypic classes (Fig. 6). The majority produced parental or undetectable levels of antibiotic whilst two recombinants, *P. fluorescens*::33.2:5.4 and *P. fluorescens*::210.1:44.1 (see legend to Fig. 6 for explanation of these names), produced intermediate levels. Recombinants that produced parental levels of antibiotic most probably resulted from integration within an intergenic region or in a region that extended across a boundary of a gene. Those recombinants producing reduced or undetectable levels of antibiotic were presumed to have arisen from integration into a gene involved in pseudomonic acid biosynthesis. The intermediate phenotype of *P. fluorescens*::33.2:5.4 and *P. fluorescens*::210.1:44.1 could be explained if integration resulted in slight truncation of a gene, or if a gene was expressed from a vector promoter (i.e. *Plac* of pCAW5.2). Alternatively, disruption of some genes could still allow the accumulation of a partially active intermediate of the pseudomonic acid biosynthetic pathway.

Integrations that blocked antibiotic activity were identified up to 8 kb from *mup*-33 and 7 kb from *mup*-210. This suggested that pseudomonic acid biosynthetic genes extend between *mup*-33 and *mup*-210. Whether biosynthetic genes traverse the entire region should be elucidated following gene disruption across the 5 kb of DNA unique to λ CAW210.17. Inactivation of antibiotic activity was also possible using DNA up to the end of λ CAW33.2. Thus it is probable that biosynthetic genes extend beyond the limits of λ CAW33.2.

A gene known to confer resistance to pseudomonic acid A is not clustered with biosynthetic genes in *P. fluorescens* NCIB 10586

Two plasmids capable of conferring resistance to pseudomonic acid on *E. coli* were identified in the genomic library in pAT153, following replication of the library on to medium containing pseudomonic acid at 500 μ g ml⁻¹. One plasmid, pBROC128, complemented the *E. coli* *ileS* temperature-sensitive mutant 331c⁻ (Isaksson *et al.*, 1977), indicating that it encoded a *P. fluorescens* isoleucyl-tRNA synthetase, known from previous studies to be inherently resistant to pseudomonic acid (Hughes & Mellows, 1980). This observation has been confirmed following DNA sequencing of the 5.8 kb insert of pBROC128 (Isaki *et al.*, 1990). The second plasmid, pBROC132, contained a 21.3 kb insert. Using a combination of Tn1723 muta-

genesis and subcloning, the relevant DNA was located to a 1.6 kb *Bam*HI fragment of pBROC132. The mechanism of resistance encoded by this locus, denoted *mupR*, is currently unresolved.

On the premise that resistance genes are generally clustered with biosynthetic genes in bacteria, it was anticipated that *mupR* could map close to one of the Tn5 insertions inactivating pseudomonic acid biosynthesis. To determine whether this were the case, *Ase*I- and *Aff*II-digested DNA from the Tn5 mutants was probed with the 1.6 kb *Bam*HI fragment. A 100 kb *Ase*I fragment and 50 kb *Aff*II fragment hybridized in each case (data not shown). Neither of these fragments corresponded to a fragment in which a Tn5 insertion mapped.

Since it was possible that *mupR* mapped alongside biosynthetic genes for which mutants are not available, gene disruption analysis was also performed around *mupR*. *Sau*3AI fragments obtained from DNA extending 3.5 kb in one direction and 8 kb in the other direction from *mupR* were subcloned into pCAW5.2, and individual derivatives were conjugally transferred to *P. fluorescens* NCIB 10586. Amongst a total of eight recombinants, none had any defect in pseudomonic acid production, as assessed by bioassay (data not shown). It would therefore seem unlikely that *mupR* is clustered with pseudomonic acid A biosynthetic genes.

DISCUSSION

This work has described the identification and preliminary characterization of a 60 kb region of the *P. fluorescens* NCIB 10586 chromosome that is involved in pseudomonic acid biosynthesis. Using both transposon mutagenesis and a gene-disruption approach, mutations mapping at sites throughout this region have been shown to impair antibiotic production by *P. fluorescens* NCIB 10586. For the seven Tn5 mutants that map here, this has been proved to be associated with a failure to accumulate pseudomonic acid, following HPLC analysis of culture extracts. Although the same information is not available for the gene disruption mutants, their location with respect to the Tn5 insertions strongly suggests that they too block pseudomonic acid production.

In addition to the genes mapping within the 60 kb region, at least three more genetic loci contributing to pseudomonic acid production have been identified by Tn5 mutations mapping at disparate sites on the *P. fluorescens* chromosome. Preliminary results have indicated that two Tn725 mutations (mutants 22 and 43) may also map at different sites. The amount of DNA associated with pseudomonic acid production at each of these sites is not known. As with the other transposon mutants, HPLC analysis has demonstrated that these mutants do not accumulate pseudomonic acid. It is possible that some of the mutated genes play an indirect role in pseudomonic acid production, for example in the supply of precursors to the biosynthetic genes: acetate and methionine metabolism could be relevant in this respect. Alternatively,

some of the mutations could disrupt control circuits possibly involved in the activation of more idiosyncratic aspects of *P. fluorescens* metabolism. In this respect, it was demonstrated that mutations in a gene encoding glucose dehydrogenase could prevent the activation of biosynthesis of an antifungal compound by *P. fluorescens* HV37a (Gutterson *et al.*, 1988). The similarities between polyketide and fatty acid biosynthesis may also entail some enzymes being common to the two processes. However, such possibilities may be qualified by the fact that none of the mutants display obvious physiological defects (including growth rate, morphological appearance, growth requirements, or accumulation of abnormal compounds detectable by the HPLC analysis) aside from the failure to accumulate pseudomonic acid. Interestingly, a gene that encodes resistance to pseudomonic acid, *mupR*, has been mapped to a site distinct from any of the loci involved in pseudomonic acid production. The significance of this observation awaits demonstration of the role of *mupR* in *P. fluorescens* NCIB 10586; attempts to identify the phenotype of a mutant carrying a defective copy of *mupR* have so far proved unsuccessful.

Further analysis of pseudomonic acid biosynthesis will require characterization of genes within the biosynthetic cluster. Identification of the biosynthetic defects in the non-producing mutants will be helpful in this respect. In an attempt to identify possible homologues of known polyketide synthase genes, chromosomal DNA of *P. fluorescens* NCIB 10586 has been probed with DNA from the actinorhodin, granaticin and tetracenomycin clusters (Malpartida & Hopwood, 1986; Sherman *et al.*, 1989; Bibb *et al.*, 1989; DNA kindly provided by M. J. Bibb) and also with oligonucleotides designed on the basis of conserved domains within β -ketoacyl synthases. A sequence hybridizing to an *actI* probe has been identified, but awaits characterization. Random DNA sequence analysis throughout the 60 kb region is also being performed, with the aim of finding ORFs that could encode components of a polyketide synthase.

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