Mutational Analysis Reveals That All Tailoring Region Genes Are Required for Production of Polyketide Antibiotic Mupirocin by Pseudomonas fluorescens

PSEUDOMONIC ACID B BIOSYNTHESIS PRECEDES PSEUDOMONIC ACID A 

Received for publication, February 20, 2007, and in revised form, March 21, 2007 Published, JBC Papers in Press, March 23, 2007, DOI 10.1074/jbc.M701490200

Joanne Hothersall, Ji’en Wu, Ayesha S. Rahman, Jennifer A. Shields, James Haddock, Nicola Johnson, Sian M. Cooper, Elton R. Stephens, Russell J. Cox, John Crosby, Christine L. Willis, Thomas J. Simpson, and Christopher M. Thomas

From the ‡School of Biosciences, University of Birmingham, Edgbaston, Birmingham B15 2TT, United Kingdom and the §School of Chemistry, University of Bristol, Cantock’s Close, Bristol BS8 1TS, United Kingdom

The Pseudomonas fluorescens mupirocin biosynthetic cluster encodes six proteins involved in polyketide biosynthesis and 26 single polyketides proposed to perform largely tailoring functions. In-frame deletions in the tailoring open reading frames demonstrated that all are required for mupirocin production. A bidirectional promoter region was identified between mupF, which runs counter to other open reading frames and its immediate neighbor macpC, implying the 74-kb cluster consists of two transcriptional units. mupD/E and mupH/K must be cotranscribed as pairs for normal function implying co-assembly during translation. MupJ and K belong to a widely distributed enzyme pair implicated, with MupH, in methyl addition. Deletion of mupF, a putative ketoreductase, produced a mupirocin analogue with a C-7 ketone. Deletion of mupC, a putative dienoyl CoA reductase, generated an analogue whose structure indicated that MupC is also implicated in control of the oxidation state around the tetrahydropyran ring of monic acid. Double mutants with ΔmupC and ΔmupO, ΔmupU, ΔmupV, or ΔmacpE produced pseudomonic acid B but not pseudomonic acid A, as do the mupO, U, V, and macpE mutants, indicating that MupC must work after MupO, U, and V.

Mupirocin is a polyketide antibiotic active against Gram-positive bacteria, including methicillin-resistant Staphylococcus aureus. It is a competitive inhibitor of isoleucyl-tRNA synthetase (IleRS), blocking binding of isoleucine and ATP to IleRS (1). Mupirocin is a mixture of pseudomonic acids (PA) produced by Pseudomonas fluorescens NCIMB 10586, with PA-A accounting for 90%. PA-A consists of a C9 saturated fatty acid (9-hydroxynonanoic acid, 9HN) joined via an ester linkage to a C17 unsaturated polyketide moiety containing a tetrahydropyran ring (monic acid, MA, Fig. 1A) (2, 3). PA-B has an additional hydroxyl group at C-8 (4), in PA-C the C-10,11 epoxide group is replaced with a double bond (5), and PA-D has an additional double bond at C-4',5' in the 9-HN moiety (6).

Polyketides are biosynthesized by condensation of small carboxylic acids catalyzed by ketosynthases (KS) generating β-carbonyl groups that may then be left unreduced or reduced partially or fully by the combined activities of ketoreductases (KR), dehydratases (DH), and enoyl reductases (ER). In archetypal Type I polyketide synthase (PKS) systems the starter and extender carboxylic acids are loaded onto KS and acyl carrier proteins (ACP), respectively, via acyl-CoA-activated intermediates, catalyzed by acyltransferases (AT). The growing polyketide chain remains attached to an ACP as a thiolester until its release, typically by hydrolysis catalyzed by a terminal thioesterase (TE). The polyketide backbone may then require further modification to produce the final metabolite. Post-PKS tailoring includes such processes as further oxidative-reductive steps, addition of moieties such as sugar units, methylation, halogenation, and cyclization. It is this variety in reduction, addition of functional groups, and choice of carboxylic units that make the polyketides a very diverse class of metabolites in both structure and activity (7). There are several examples of PKS combinatorial biosyntheses resulting in novel polyketide metabolites (for a review see Ref. 8), and such an approach is also being applied to tailoring enzymes (for a review see Ref. 9).

The 74-kb mupirocin biosynthetic gene cluster has been sequenced and many of the open reading frames (ORFs) assigned putative functions (Table 1 and Fig. 2) (10). The first half of the cluster contains 3 large Type I multifunctional PKS genes plus associated trans-acyltransferase as well as two single ORFs mupA and mupB, while the second half, referred to as the tailoring region, contains 26 single ORFs (mupC-X, and

thioesterase; MeT, methyltransferase; HMG, 3-hydroxy-3-methylglutaric acid; PPTase, phosphopantetheinyl transferase; N-AHL, N-acyl homoserine lactone; IPTG, isopropyl-1-thio-β-D-galactopyranoside; HPLC, high pressure liquid chromatography.

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 282, NO. 21, pp. 15451–15461, May 25, 2007 © 2007 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in the U.S.A.
Mupirocin Biosynthesis Tailoring Region

mcpa-E), plus two PKS-like genes mppe and mmpf. A model for biosynthesis of the mupirocin backbone was proposed based on the cluster sequence, feeding experiments (11, 12) and comparisons with other polyketide biosynthetic pathways (Fig. 2B) (10). MmpD and MmpA together contain 6 modules for condensation and reduction of acetate-derived units, which with two methyl transferase domains and assuming that the DH domain in module 1 is inactive (because it contains aspartic acid and alanine residues instead of glycine and proline in the DH motif HXXXGXXX) can generate the backbone of a C16-heptaketide MA precursor. The source of 9-HN is more difficult to predict. A 3-hydroxypropionate starter unit may be extended by three malonate condensations, possibly catalyzed by a dedicated fatty acid synthase (FAS), or MmpB functioning iteratively with additional ER activity perhaps provided by MupE and/or MupD. The resulting backbone would need further modifications to produce the major metabolite PA-A shown in Fig. 1A, either prior to or after esterification of MA and 9-HN. These modifications include oxidation of the C-10,11 double bond to create the C-10,11 epoxide group, C-8,9 double bond reduction and C-16 oxidation to produce the tetrahydropyran ring, C-6 hydroxylation, and incorporation of the C-15 methyl group derived from a cleaved acetate unit. We recently reported that mutagenesis of MupW, a putative dioxygenase, generates a novel PA metabolite, mupirocin W, lacking the tetrahydropyran ring (13). Thus MupW appears to be responsible for oxidative activation of the C-16 methyl group required for the formation of the tetrahydropyran ring, while esterification of MA or an immediate precursor with 9-HN and other tailoring modifications can occur without tetrahydropyran ring formation. Therefore either pyran ring formation occurs after esterification, or there is relaxed substrate specificity of the enzymes involved in these post-PKS modifications. In the same study mutagenesis of any one of four other ORFs mupo, mupu, mupv, and mcpE resulted in a complete switch to PA-B as the major metabolite and so showed that they are essential for PA-A production but not PA-B (14). This implies that PA-B is not simply formed, as might have been expected, by facile hydroxylation of PA-A, but is instead either a precursor to PA-A or more likely a shunt product.

Our previous work also reported deletion of mupQ, S, T, R, X, and I, of which only mupR and I could be assigned a clear role in quorum regulation of expression of the cluster (10, 14). This study reports the in-frame deletion analysis of all remaining ORFs in the mupc-mupx tailoring region showing that all are indeed required for PA production. Furthermore HPLC analysis of the PA profile of these mutants compared with wild-type NCIMB 10586, revealed a number of novel shunt products, exemplified by mupirocin C and mupirocin F shown in Fig. 1, B and C. The role of specific proteins in mupirocin biosynthesis is discussed here while details of structural characterization of these and other novel metabolites will be reported in full elsewhere.

TABLE 1

| ORF  | Deduced function                              | ORF  | Deduced function                              |
|------|----------------------------------------------|------|----------------------------------------------|
| mupa | FMNH, oxygenase                               | mmpE | PKS: KS, hydroxylase                          |
| mupA | PKS: KS, ACP, KS, KR, ACP, KS, ACP            | mupL | Hydrolase                                     |
| mupB | 3-oxo-ACP synthase                           | mupM | Isoleucyl-tRNA synthetase                    |
| mupC | PKS: KS, DH, KR, ACP, ACP, ACP                | mupO | Cytochrome P450                               |
| mupD | PKS: KS, DH, Me, ACP, KS, DH, KR, ACP, KS,    | mupP | Acyl-CoA synthase                             |
|      | DH, KR, Me, ACP, KS, KR, ACP                  | mupQ | 3-oxo-ACP reductase                           |
| mupC | Diamyl-CoA reductase                          | mupS | ACP                                          |
| mupA | ACP                                          | mupD | PKS: KS                                       |
| mupD | PKS: KS, ACP, KR, ACP                         | mupF | ACP                                          |
| mupE | Enoyl reductase                               | mupE | PKS: KS                                       |
| mupA | ACP                                          | mupE | ACP                                          |
| mupD | 3-oxo-ACP reductase                           | mupE | ACP                                          |
| mupG | 3-oxo-ACP synthase                            | mupF | Ferredoxin dioxygenase                        |
| mupH | HMG-CoA synthase                              | mupG | Ferredoxin dioxygenase                        |
| mupJ | Enoyl-CoA hydratase                           | mupH | Acyl-CoA synthase                             |
| mupK | Enoyl-CoA hydratase                           | mupI | Oxidoreductase                                |

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids—P. fluorescens NCIMB 10586 (15) was used as the wild-type mupirocin producer. Escherichia coli strain DH5α (16) was used for plasmid
transformation and propagation; S17-1 (17) was used to mobilize suicide and expression plasmids into \textit{P. fluorescens}. \textit{Bacillus subtilis} 1064 (18) was used in bioassays to monitor mupirocin production. PCR fragments were initially cloned into pGEM-TEasy (Promega) for sequencing. Other plasmids and bacterial strains used or constructed in this study are listed in Tables 2 and 3.

**Growth and Culture Conditions**—\textit{E. coli} strains were grown at 37 °C in L-broth (19) and L-agar (L-broth supplemented with 1.5%, w/v agar) supplemented with appropriate antibiotics. \textit{P. fluorescens} strains were grown at 30 °C in L-broth and L-agar. For plasmid maintenance and selection of antibiotic-resistant transformants media was supplemented with appropriate antibiotic concentrations as follows: ampicillin (100 \( \mu \)g/ml), kanamycin (50 \( \mu \)g/ml) and tetracycline-HCl (15–25 \( \mu \)g/ml).

**DNA Isolation and Manipulation**—Plasmid DNA extraction was performed by the alkaline SDS method of Birnboim and Doly (20), or with Wizard Plus SV Mini Preps DNA Purification Systems (Promega). Extraction of DNA from agarose was performed using GeneClean kit (Bio101). Ligations were performed using T4 DNA ligase (21). PCR fragments were cloned into a T-tailed vector pGEM-TEasy (Promega). Competent \textit{E. coli} cells were transformed with plasmid DNA using the method of Cohen et al. (22).

**Sequence Analysis**—DNA sequencing was carried out using the Big Dye Terminator kit (PE-ABI), which is based on the chain termination method (23). The sequencing reactions were separated on an ABI 3700 DNA Analyzer.

**Suicide Mutagenesis of \textit{P. fluorescens}**—Bi-parental mating was carried out to mobilize the suicide plasmid derivatives and expression vectors from \textit{E. coli} S17-1 to \textit{P. fluorescens} as follows. A mixture of late exponential phase cultures of \textit{E. coli} S17-1, containing the relevant plasmid (0.5 ml), and \textit{P. fluorescens} (0.5 ml) were filtered on to a 0.45-\( \mu \)m sterile Millipore filter. The filter was placed on L-agar overnight at room temperature. The mating mixture was resuspended in sterile saline solution (1 ml), and aliquots (100 \( \mu \)l) were spread on M9 minimal medium or L-agar supplemented with the appropriate antibiotic to select for the presence of the plasmid and not support \textit{E. coli} S17-1 growth. To isolate strains in which the suicide plasmid had excised from the chromosomal DNA, the co-integrant clone was incubated in L-broth at 30 °C without antibiotic selection. Serial dilutions were plated on L-agar supplemented with sucrose (5%) and colonies replica plated onto L-agar Km. Colonies that were Suc' and Km' were selected.

**Bioassay for Mupirocin Production**—10-\( \mu \)l samples of 16 h \textit{P. fluorescens} cultures, diluted to the same A\_600, were spotted onto L-agar and incubated at 30 °C for 16 h. The bioassay plates were then overlaid with molten L-agar seeded with a 16 h \textit{B. subtilis} culture (150 \( \mu \)l/ml) and triphenyl tetrazolium chloride (0.025%), incubated at 37 °C for 16 h and scored for the presence of clear zones around the \textit{P. fluorescens} patch.

**HPLC Analysis of PA**—Overnight MPM (2.3 g liter\(^{-1}\) yeast extract, 1.1 glucose, 2.6 Na\(_2\)HPO\(_4\), 2.4 KH\(_2\)PO\(_4\), 5.0 (NH\(_4\))\(_2\)SO\(_4\)) seed cultures incubated at 25 °C, 200 rpm were diluted 20-fold into 25 ml MPM and incubated for 40 h, at 22 °C, 200 rpm. 1-ml aliquots were centrifuged at 15,000 \( \times \) g for 10 min, and the supernatant stored at \(-20^\circ\)C. Samples were filtered (0.2 \( \mu \)m) prior to analysis. HPLC was performed using either Gilson 712 or Unipoint LC system software, reverse phase C18 column (15 cm \( \times \) 4.6 mm), UV detection at 233 nm, and mobile phase water/acetonitrile gradient (5–70% acetonitrile trifluoroacetic acid (0.01%)) over 30 min at 1 ml/min flow rate.

![FIGURE 2. A, summary of the mupirocin biosynthesis cluster. B, scheme for biosynthesis of monic acid precursor. C, scheme for incorporation of a C-15 methyl group by MupH, J, and K.](image-url)
HPLC Purification of Novel PA Intermediates—

10586ΔmupC and 10586ΔmupF were cultured as described previously for HPLC analysis. 400-ml culture was centrifuged at 4500 g, 25 °C for 10 min. The supernatant was acidified with HCl to pH 4.5 then solvent extracted with ethyl acetate (2:1). The aqueous layer was re-extracted twice. The solvent layers were pooled, filtered over anhydrous MgSO₄, then dried by rotary evaporation. The crude extract was resuspended in 2 ml of methanol.

### TABLE 2
Details of plasmids used for gene knockout and complementation experiments

| Plasmid    | Description                                                                 | Source    |
|------------|------------------------------------------------------------------------------|-----------|
| pAKE604    | 5.9 kb, pMB1 replicon, Ap<sup>+</sup>, oriT, lacZa, sacB                     | This study|
| pASRA01    | pAKE604 + BamHI-EcoRI 1150 bp PCR fragment, Δ225 bp in macpA                | This study|
| pASRA11    | pJH110 + EcoRI-Xbal 300 bp macpA PCR fragment                               | This study|
| pASRB01    | pAKE604 + BamHI-EcoRI 1070 bp PCR fragment, Δ198 bp in macpB                | This study|
| pASRB11    | pJH110 + EcoRI-Xbal 250 bp macpB PCR fragment                               | This study|
| pASRC01    | pAKE604 + BamHI-EcoRI 1030 bp PCR fragment, Δ183 bp in macpC                | This study|
| pASRC11    | pJH110 + EcoRI-Xbal 230 bp macpC PCR fragment                               | This study|
| pASRD01    | pAKE604 + BamHI-EcoRI 1050 bp PCR fragment, Δ285 bp in macpD                | This study|
| pASRD11    | pJH110 + EcoRI-Xbal 320 bp macpD PCR fragment                               | This study|
| pJH10      | 14.5 kb, IncQ replicon, Te<sup>+</sup>, Sm<sup>+</sup>, oriT, tacz, lacZ<sup>+</sup> | (10)      |
| pJHC01     | pAKE604 + BamHI-Xbal 1098 bp PCR fragment, Δ1236 bp in mupC                 | This study|
| pJHC11     | pJH110 + EcoRI-Xbal 1308 bp mupC PCR fragment                               | This study|
| pJHD01     | pAKE604 + BamHI-EcoRI 1053 bp PCR fragment, Δ693 bp in mupD                 | This study|
| pJHD11     | pJH110 + EcoRI-Xbal 750 bp mupD PCR fragment                                | This study|
| pJHDE01    | pAKE604 + BamHI-EcoRI 1036 bp PCR fragment, Δ1733 bp in mupD and mupE       | This study|
| pJHDE11    | pJH110 + EcoRI-Xbal 1802 bp mupD mupE PCR product                           | This study|
| pJHE01     | pAKE604 + Sall-HindIII 1011 bp PCR fragment, Δ951 bp in mupE                | This study|
| pJHE11     | pJH110 + EcoRI-Xbal 1035 bp mupE PCR fragment                               | This study|
| pJHF01     | pAKE604 + BamHI-EcoRI 1025 bp PCR fragment, Δ933 bp in mupF                  | This study|
| pJHF11     | pJH110 + EcoRI-Sacl 1027 bp mupF (reverse) PCR fragment                     | This study|
| pJHF12     | pJH110 + EcoRI-KpnI 1397 bp mupF (forward) macpC PCR fragment               | This study|
| pJHF13     | pJH110 + EcoRI-Xbal 1421 bp macpC mupF (reverse) PCR fragment               | This study|
| pJHF14     | pJH110 + EcoRI-Xbal 1421 bp mupF (forward) macpC PCR fragment               | This study|
| pJIG01     | pAKE604 + HindIII-BamHI 1008 bp PCR fragment, Δ1113 bp in mupG               | This study|
| pJIG11     | pJH110 + EcoRI-Xbal 1251 bp mupG PCR fragment                               | This study|
| pJHL01     | pAKE604 with BamHI-EcoRI 1024 bp PCR fragment, Δ858 bp in mupL              | This study|
| pJHL11     | pJH110 + EcoRI-Xbal 951 bp mupL PCR fragment                                | This study|
| pJHM01     | pAKE604 + EcoRI-Xbal 1064 bp PCR fragment, Δ3057 bp in mupM                 | This study|
| pJHM11     | pJH110 + EcoRI-Xbal mupM, generated from EcoRI-KpnI PCR fragment, KpnI-Sal 1.87 kb pAKE900 and Sall-Xbal PCR fragment | This study|
| pJHN01     | pAKE604 + BamHI-EcoRI 1011 bp PCR fragment, Δ735 bp in mupN                 | This study|
| pJHN11     | pJH110 + EcoRI-Xbal 846 bp mupN PCR fragment                                | This study|
| pJHP01     | pAKE604 + Sall-EcoRI 1036 bp PCR fragment, Δ603 bp in mupP                  | This study|
| pJHP11     | pJH110 + KpnI-Sacl 942 bp mupP forward ORF PCR fragment                     | This study|
| pJHP12     | pJH110 + KpnI-Sacl 807 bp mupP reverse ORF PCR fragment                     | This study|
| pJSΔJ      | pAKE604 with BamHI-EcoRI 995 bp PCR fragment, Δ632 bp in mupJ               | This study|
| pJSJ       | pJH110 + EcoRI-Xbal 766 bp mupJ PCR fragment                                | This study|
| pJSΔJK     | pAKE604 + BamHI-EcoRI 1023 bp PCR fragment, Δ1449 bp in mupJ and mupK       | This study|
| pJSJK      | pJH110 + EcoRI-Xbal 1562 bp mupK mupK PCR fragment                          | This study|
| pJSΔK      | pAKE604 + BamHI-EcoRI 1047 bp PCR fragment, Δ633 bp in mupK                 | This study|
| pJSK       | pJH110 + EcoRI-Xbal 744 bp mupK PCR fragment                                | This study|
| pJSCSA      | pAKE604 + BamHI-EcoRI 1008 bp PCR fragment, Δ784 bp in macpC point mutation aa S38A | This study|
| pKS9CA      | pAKE604 + BamHI-EcoRI 1020 bp PCR fragment mmpES9 point mutation C215A      | This study|
| pKS10CA     | pAKE604 + BamHI-EcoRI 1035 bp PCR fragment, mmpES9 point mutation aa C183A, plus nt A63899G and T63902C generated SacI site | This study|
| pSCH        | pAKE604 + EcoRI-Xbal 1.2 kb PCR fragment, Δ984 bp in mupH                  | This study|
| pSCCH       | pJH110 + EcoRI-Sacl 1.3 kb mupH PCR fragment                                | This study|
Diluted aliquots were filtered (0.2 μm) and analyzed by HPLC as described previously with varying mobile phase conditions to determine maximal separation of the peak of interest. Fractions were collected from a protracted isocratic elution step using the water/acetonitrile % calculated, pooled, and re-separated under the same conditions. Fractions were again collected, pooled, and dried by rotary evaporation. The purified extract was resuspended in 1 ml of methanol.

ES-MS—PA intermediates were analyzed by ES-MS on a Kratos Profile Mass Spectrometer.

Determining MIC of Mupirocin—The cell viability of Pseudomonas strains incubated at 30 °C, 200 rpm for 16 h was determined. Using these values repeat cultures were diluted to 0.5 × 10⁸ cell/ml 1.1 × 10⁶ cells were spotted onto LA containing 0–2000 μg/ml mupirocin and incubated at 30 °C for 2 days. The reaction was heat-inactivated at 70 °C for 15 min. 10% of the first strand cDNA mix was then used as template in the second strand synthesis step using Invitrogen DNA Taq polymerase (2 units), two primers (0.2 M) designed to generate 450-bp fragments (primers: RTmupF & DNAmupF: caggtcgcgcacatccac, RTmupG & DNAmupG: cagtgggcagatggcgat, RTmupP & DNAmupP: cgagggctggcacgc, RTmupO & DNAmupQ: ccacagttgcctcagcctc).

RESULTS

All ORFs of the mup Tailoring Region Are Required for Normal Mupirocin Production—The remaining ORFs of the mup tailoring region were targeted, creating in-frame deletions, and attempting to complement them as described (14). The process is illustrated by detailed description of mupC. An in-frame deletion of mupC was created by amplifying two DNA fragments, mupC1 and mupC2, which flank the region to be deleted. The 588-bp mupC1 fragment generated from primers mupC1F and mupC1R includes the first 27 bp of mupC while the 516-bp mupC2 fragment generated from primers mupC2F and mupC2R includes the final 33 bp (for primer

| Strain       | Genotype  | Bioassay | HPLC Peak Area PA-A (% WT) | Complementation | Bioassay  |
|--------------|-----------|----------|-----------------------------|-----------------|-----------|
| 10586ΔmupC*  | mupCA10-421 | 23 ± 3 (n=16) | 6 ± 4 (n=4) | pH1C1 | 64 ± 16 (n=6), 0 mM IPTG |
| 10586ΔmupDA | mupGA24-88  | 7 ± 0.2 (n=3) | 36 ± 6 (n=5) | pH1D1 | 57 ± 7 (n=4), 0 mM IPTG |
| 10586ΔmupD  | mupDA9-239  | 63 ± 7 (n=14) | nd | pHDE11 | 48 ± 20 (n=3), 0 mM IPTG |
| 10586ΔmupE  | mupEA14-330 | 25 ± 4 (n=14) | 11 ± 4 (n=3) | pHIE1 | 20 ± 6 (n=3), 0 mM IPTG |
| 10586ΔmupF  | mupF9-1319  | 40 ± 9 (n=29) | 2 ± 4 (n=4) | pASR11 | 78 ± 0.45 (n=3), 0.5 mM IPTG |

* Deleted amino acid co-ordinates relative to ORF (inclusive).
* Area of zone of inhibition minus area of colony, expressed as percentage of WT, (nd) nothing detectable, (–) not determined, (**) double mutants of mupC with mupO, U, V or mupE were phenotypically as for single mutants of mupO, U, V and mupE as previously described (14).

**TABLE 3**

Summary of gene deletion and complementation experiments

| Strain       | Genotype  | Bioassay | HPLC Peak Area PA-A (% WT) | Complementation | Bioassay  |
|--------------|-----------|----------|-----------------------------|-----------------|-----------|
| 10586ΔmupCSA | mupCS38A  | 3 ± 2 (n=2) | nd | nd | 29 ± 18 (n=4), 0.1 mM IPTG |
| 10586ΔmupE  | mupGA27-397 | 8 ± 7 (n=13) | 8 ± 7 (n=3) | pH1G1 | 79 ± 14 (n=4), 0.5 mM IPTG |
| 10586ΔmupH  | mupHA48-375 | 14 ± 6 (n=3) | nd | pSCH1 | 70 ± 17 (n=4), 0.1 mM IPTG |
| 10586ΔmupJ  | mupJA19-228 | 7 ± 1 (n=8) | nd | pSJC1 | 92 ± 11 (n=2), 0.1 mM IPTG |
| 10586ΔmupK  | mupKA20-230 | 3 ± 2 (n=10) | nd | pSJC2 | 9 ± 0.9 (n=3), 0.1 mM IPTG |
| 10586ΔmupL  | mupLA1-2299 | 13 ± 3 (n=8) | 1 ± 1 (n=3) | pSJC3 | 86 ± 19 (n=4), 0.1 mM IPTG |
| 10586ΔmupM  | mupMN7-1025 | 40 ± 8 (n=30) | 5 ± 7 (n=2) | pHL1 | 102 ± 4.5 (n=5), 0 mM IPTG |
| 10586ΔmupN  | mupNA23-267 | 11 ± 12 (n=11) | nd | pHJM1 | 39 ± 5 (n=4), 0 mM IPTG |
| 10586ΔmupP  | mupPA13-215 | 33 ± 3 (n=14) | 24 ± 2 (n=8) | pHN1 | 84 ± 10 (n=3), 0.1 mM IPTG |
| 10586ΔmupQ  | mupQC54-160 | 21 ± 4 (n=4) | nd | pHPI | 62 ± 3 (n=4), 0.1 mM IPTG |
| 10586ΔmupR  | mupRA14-123 | 6 ± 3 (n=6) | 2 ± 3 (n=4) | pHPI2 | 11 ± 13 (n=8), 0 mM IPTG |

**TABLE 3**

Summary of gene deletion and complementation experiments

| Strain       | Genotype  | Bioassay | HPLC Peak Area PA-A (% WT) | Complementation | Bioassay  |
|--------------|-----------|----------|-----------------------------|-----------------|-----------|
| 10586ΔmupC  | mupCA8-68  | 7 ± 2 (n=8) | nd | nd | 19 ± 28 (n=4), 0.1 mM IPTG |
| 10586ΔmupC  | mupCA8-68  | 7 ± 2 (n=8) | nd | nd | 29 ± 18 (n=4), 0.1 mM IPTG |
| 10586ΔmupC  | mupCA8-68  | 7 ± 2 (n=8) | nd | nd | 29 ± 18 (n=4), 0.1 mM IPTG |
| 10586ΔmupC  | mupCA8-68  | 7 ± 2 (n=8) | nd | nd | 29 ± 18 (n=4), 0.1 mM IPTG |

**TABLE 3**

Summary of gene deletion and complementation experiments

| Strain       | Genotype  | Bioassay | HPLC Peak Area PA-A (% WT) | Complementation | Bioassay  |
|--------------|-----------|----------|-----------------------------|-----------------|-----------|
| 10586ΔmupC  | mupCA8-68  | 7 ± 2 (n=8) | nd | nd | 29 ± 18 (n=4), 0.1 mM IPTG |
Mupirocin Biosynthesis Tailoring Region

A bioassay of eight ΔmupC mutants revealed a consistent reduction in antibacterial activity against *B. subtilis* 1064 to 23% of WT levels (Table 3 and Fig. 3A). The mupC ORF was then amplified by PCR (primers CmupCF/CmupCR) and cloned into the IncQ *tac* promoter expression vector pHJ10, giving pHJC11 (Table 2, for primer details see supplemental Table S2). The resulting plasmid was introduced into the ΔmupC strain and bioassay showed complementation (Table 3). Therefore the defect in PA production must be due to loss of mupC function and not to a polar effect on downstream genes. Conditions for ΔmupC complementation were optimal without IPTG; biological activity restored to 64% of WT levels.

In the same way, 16 further tailoring region ORFs (*mupD*, *E*, *F*, *G*, *H*, *I*, *K*, *L*, *N*, *P*, and *macpA*, *B*, *C*, and *D*, and *mupE* and *mupF*) were mutated. When analyzed by bioassay, the majority of mutants showed essentially a PA-negative phenotype with a reduction in average biological activity against *B. subtilis* 1064 to \(<20\%\) of WT levels (see Table 3 and Fig. 3A). Four mutants had less reduced levels of activity: 10586ΔmupD had 63\% activity, 10586ΔmupF 40\%, 10586ΔmupM 40\%, and 10586ΔmupP 33\%.

Again each ORF was cloned separately as a PCR-amplified fragment in pHJ10 to be expressed in *trans* to the appropriate chromosomal mutation (Table 2). Complementation of ΔmupF and ΔmupP, whose orientations had to be determined in combination with complementation, is described in the next section. Most of the other complementation strains showed increased activity in the bioassay compared with appropriate controls containing the empty vector. Exceptions to this were 10586ΔmupD(pJH11), 10586ΔmupE(pJHE11), 10586ΔmacpC(pASRC11), 10586ΔmupK(pScK), and 10586ΔmupM(pJHM11) whose levels remained the same as the mutant strains (Table 3).

MupM is an isoleucyl-tRNA synthetase previously shown to confer mupirocin resistance on *E. coli* (10). MIC determination as described under “Experimental Procedures” showed that NCIMB 10586 is resistant to mupirocin in excess of 2000 \(\mu\)g/ml while the ΔmupM strain had a MIC between 600 and 800 \(\mu\)g/ml. 10586ΔmupM(pJHM11) grew on 2000 \(\mu\)g/ml when induced with 0.1 \(\mu\)M IPTG. 10586ΔmupM(pHJ10) did not restore resistance. Thus while the deletion in *mupM* may have polar effects which disrupt mupirocin production, its ability to confer mupirocin resistance is still complementable.

The other deletions that were not complemented include four that are grouped as pairs of neighboring ORFs: *mupD* with *mupE*, and *mupF* with *mupK*. This led to the hypothesis that these gene pairs must be translated from the same mRNA to be functional. For example their nascent polypeptides may need to interact to fold into functional enzymes. To test this *mupD* and *mupE* were PCR-amplified together and cloned into pHJ10 (giving pHJDE11) (for details see Table 2). pHJDE11 was initially introduced into both 10586ΔmupD and 10586ΔmupE and tested by plate bioassay for its ability to restore mupirocin production. Complementation was only seen in the ΔmupM strain. Likewise *mupF* and *mupK* were cloned together giving pScJK, which restored mupirocin production to 10586ΔmupF but not 10586ΔmupK. One possible explanation for the continued lack of complementation in the ΔmupD or the ΔmupK single mutants was that the remaining ORF in the chromosome produces a non-functional product that interferes with the functional product(s) of the two ORFs expressed together. To test this, two double in-frame deletions were created either removing both *mupD* and *mupE*, or both *mupF* and *mupK*. Introduction of pJHE11 or pScJK, respectively, into these double mutants restored antibiotic activity to near WT levels, consistent with the above hypothesis.

To test whether macpC also required co-expression with a neighboring ORF to be functional when expressed in *trans* macpC was cloned into pHJ10 together with the upstream ORF mupF in both possible mupF orientations (pJH12 and pHJ13). pHJ12 and pHJ13 were able to complement 10586ΔmacpC and restore mupirocin production.

An Internal Promoter Is Located Upstream of macpC—Assignment of ORFs based on the sequence of the *mup* region indicated that almost all the ORFs run in the same direction, and it is tempting to suggest that the whole region from *mupA* to *mupX* might constitute a single transcriptional unit. How-
ever, two of the genes of the tailoring region, mupF and mupP, were predicted (based on codon usage and the presence of appropriate ribosome binding sites) to run in the opposite direction (10), although for both there are alternative ORFs on the opposite strand. Homology (Blast) searches did not give useful insight into which orientation is the most plausible. For mupF the reverse orientation (coordinates 45344–44334 bp) shows 28% identity with a ketoreductase from Callistephus chinensis (accession P51103) while the forward orientation (coordinates 44358–45191 bp) reveals 31% identity with cinnamoyl CoA reductase from barley Hordeum vulgare (accession AY149607). For mupP no significant alignments were observed in either the forward (coordinates 59395–60864 bp) or reverse orientations (coordinates 60615–59821 bp). We therefore performed RT-PCR on the mupF and mupP genes as well as the genes downstream of them mupG and mupQ to determine which transcription events could be detected as described under “Experimental Procedures.” Primers were designed to generate 450-bp fragments. Unfortunately in all cases several fragments were amplified and for both orientations. Nevertheless, for mupG and mupQ, which were being used as positive forward controls, only the forward orientation primers gave fragments of the correct size whose identity was confirmed by cloning and sequencing. The results were less conclusive for mupF and mupP. Fragments of the correct size were amplified from mupF transcripts in both directions. The stronger forward fragment was cloned and sequenced and again showed the appropriate region had been amplified. Several fragments of similar size were amplified from mupP transcripts again in both directions. Sequence was obtained for the most suitably sized segment was unable to complement the mutation, which was in-frame for both possible directions. Sequence was obtained for the most suitably sized fragment (coordinates 44334–45742 bp) including 21-bp—HPLC analysis was carried out on each of the in-frame deletion mutants to investigate whether they produce novel metabolites that would enable the function of their encoded protein to be determined. While all mutants showed reduced PA-A production, only 10586ΔmupC and 10586ΔmupF produced readily detectable new peaks under the conditions of growth and analysis used. WT P. fluorescens, analyzed by HPLC as described

**Mupirocin Biosynthesis Tailoring Region**

![Organism Diagram](attachment:organism.png)

**FIGURE 4. Organization of mupF and macpC expression vectors.** pJHF12 contains a segment that has the whole of the putative forward mupF ORF but truncates the reverse ORF by seven triplets. In pJHF12 this segment is orientated so that mupF would be transcribed from tacp. In pJHF13 and 14 the inserted segment is longer and includes the whole of the putative reverse mupF ORF. In pJHF14 this segment is orientated so that mupF would not be transcribed from tacp.

To further investigate the orientation of mupF, expression vectors were constructed with mupF ORFs in both orientations relative to the vector tac promoter and tested for their ability to complement 10586ΔmupF. The in-frame mupF deletion mutation had been designed for the reverse orientation of mupF; but in the forward ORF the deletion extended beyond the stop codon, although translation would be terminated in the latter by a second stop codon 36-bp downstream of the first stop codon. Because of the difficulties of complementing the ΔmacpC mutant the whole of the forward mupF ORF and adjacent macpC were amplified by PCR together as a single EcoRI/KpnI fragment (coordinates 44358–45742 bp) including 21-bp upstream of the putative valine start codon of mupF. Cloned under the control of the tac promoter in pJH10 (pJHF12) this segment was unable to complement the ΔmupF mutation in trans although it could partially complement 10586ΔmacpC. Consequently a new segment (coordinates 44334–45742 bp) that includes the whole of the ORF representing mupF if it runs in the reverse orientation was amplified and cloned as an EcoRI/XbaI fragment into pH10, again together with adjacent macpC and their intergenic region. Two such expression vectors were constructed so that only one ORF either mupF or macpC (pJHF13 and pJHF14 respectively, Fig. 4) should be expressed from the external tac promoter. Both these plasmids were able to complement the mupF and macpC deletions implying that mupF and macpC each has its own promoter (Fig. 3B). 10586ΔmupF was also complemented by the reverse mupF ORF expressed in trans from pH11 on its own without macpC.

Because the above results appear to rule out that the lack of complementation of ΔmupC by pASRC11 (tacp-macpC) is due to a requirement of mupF and macpC to be co-transcribed, we introduced a point mutation into the active site of MacpC (S38A, so that it would not be phosphopantetheinylated) in case the in-frame deletion had a secondary effect such as reduction of mupF expression. This mutation resulted in complete loss of mupirocin production as indicated by both plate bioassay and HPLC analysis of culture supernatant, but could be complemented by pASRC11.

To further investigate the orientation of mupP, expression vectors were constructed with PCR-amplified mupP ORFs cloned in both orientations into pH10 (pJH11 and pJH12, Table 2). These were tested for complementation of the 10586ΔmupP mutation, which was in-frame for both possible orientations of mupP and reduced biological activity to 33% of WT. The forward direction mupP ORF when expressed from pH11 with 0.1 mM IPTG induction complemented 10586ΔmupP, as tested by bioassay, while the reverse ORF (pJH12) did not (Table 3 and Fig. 3B). This was confirmed by HPLC analysis. The peak area of PA-A from 10586ΔmupP-(pJH10) was 8 ± 4% (n = 4) relative to WT, while expressing the forward ORF in trans from pH11 increased the peak area to 156 ± 94% (n = 4). Expressing the reverse ORF in trans from pH12 did not increase the PA-A peak area, which remained low at 21 ± 17% (n = 8).

MupC and MupF Are Required for PA-A Production—HPLC analysis was carried out on each of the in-frame deletion mutants to investigate whether they produce novel metabolites that would enable the function of their encoded protein to be determined. While all mutants showed reduced PA-A production, only 10586ΔmupC and 10586ΔmupF produced readily detectable new peaks under the conditions of growth and analysis used. WT P. fluorescens, analyzed by HPLC as described.
under “Experimental Procedures,” yielded a peak with retention time 19.2 ± 0.1 min (n = 6), which has previously been identified by mass spectrometry to be PA-A (14). PA-A standard has a retention time of 19.2 min. A second smaller peak with retention time 18.4 ± 0.1 min (n = 6) is also known to be due to PA-B. HPLC analysis of 10586ΔmupC revealed a greater than 10-fold reduction in the PA-A peak area compared with WT, and the appearance of a new peak with retention time 22.1 ± 0.4 min (n = 3) (Fig. 5).

The HPLC profile of 10586ΔmupF revealed that PA-A production was negligible at 2% compared with WT. However, it also produces a number of novel minor peaks with longer retention times than PA-A (Fig. 5). Increased production, purification and identification of these intermediates will be reported separately. HPLC traces of the other mutants simply showed changes in the level of PA-A and PA-B production (Table 3). 10586ΔmupE, ΔmupG, ΔmupH, ΔmupI, ΔmupJ, mupF, mupE, mupC, mupF, mupO, mupCΔmupU and mupP were solvent extracted as described under “Experimental Procedures” and purified by HPLC. Mass spectrometry indicated a molecular weight of 496, compared with 500 for PA-A for mupirocin C, and 498 for the most abundant novel product from ΔmupF, mupirocin F. The structures (Fig. 1, B and C) were elucidated by NMR analysis (detailed description of the determination of these structures and those of metabolites isolated from other mutant strains will be reported in detail elsewhere). The structures of mupirocin F and mupirocin C are consistent with those of novel PA analogues with respectively one and two double bond equivalents more than PA-A as required by the molecular weights indicated by mass spectrometry. To further elucidate the stage where MupC is required we created double mutants in which both mupC and either mupO, mupI, mupV, or macpE were inactivated by in-frame deletion. By bioassay, HPLC profile (Fig. 5) and NMR analysis of the new peak, these double mutants behaved phenotypically like the mupO, U, V, and macpE mutants, which all accumulate PA-B instead of PA-A (14) indicating that MupC works after the products of these genes.

**DISCUSSION**

In combination with our previous work (14) the in-frame deletion analysis of the mup cluster tailoring region described here indicates that all 26 tailoring ORFs are required for normal mupirocin production. MupM activity as a eukaryotic type isoleucyl-tRNA synthetase (10, 24) should not be involved directly in PA biosynthesis so the reduction in antibacterial activity was surprising. Therefore it was of interest that mupirocin production was not restored to normal levels by expression *in trans* of mupM from pHJM11 even though its ability to confer mupirocin resistance was complemented. The reduction in mupirocin production of 10586ΔmupM may be due to polar effects on downstream genes although most of the other deletions could be complemented, suggesting that the sort of in-frame deletion mutations that we have created do not normally have polar effects. An alternative explanation would be that MupM normally forms part of a multi-protein complex involved in biosynthesis. This could have evolved as a safeguard to prevent antibiotic production in the absence of the resistance mechanism.

The presence of multi-protein complexes is also implied by the complementation tests on deletions in mupD/E and mupI/K. The fact that deletions in none of these four ORFs could be complemented by single genes *in trans*, but double deletions could be complemented by double expression plasmids, implies that these genes need to be expressed together. The lack of complementation of single gene deletions that leave behind either mupE or mupI without their partner gene in the...
chromosome implies that the products of these genes not only enter a non-functional state when expressed alone, but also block the interactions with further partners. It should therefore be useful in further work to test for these predicted protein-protein interactions. Interestingly, searches for genes related to mupI and mupK, which have related biochemical functions (enoyl-CoA hydratase), revealed many similar pairs of genes, suggesting that they might form a functional heteromultimer. For example the closest homologues of MupI and MupK are PksH (P40805) and PksI (P40802) of B. subtilis subsp. subtilis str. 168, which occur as adjacent ORFs within a polyketide biosynthesis cluster of previously unknown function (25). A closely related homologous cluster in Bacillus amyloliquefaciens has recently been assigned to bacillaene (26), and the structure of bacillaene isolated from B. subtilis has now been established so allowing an annotation of the biosynthetic gene cluster to be proposed (27). Furthermore the mupI and K enoyl-CoA hydratase gene pair is also associated with an ACP, KS, and HMG-CoA synthase, as it is in the B. subtilis cluster and in the curacin A and jamaicamide A clusters of Lyncya majusculae (28, 29). This gene cassette is proposed to introduce a methyl from acetate onto a polyketide backbone. The enoyl-CoA hydratase pair CurE and CurF from the curacin A cluster have been shown to catalyze successive dehydration of (S)-HMG-ACP to 3-methylglutaryl-ACP and decarboxylation to 3-methylcrotonyl-ACP (28). This in vitro work has been extended (30) to the full set of related genes from B. subtilis where the ACP has been shown to be converted to the corresponding malonyl-ACP by a specific malonyl transferase (PksC), decarboxylated to acetyl-ACP by the KS (PksK), which then condenses with an ACP-bound acetoacetate to give HMG-ACP (Pgs G) which is then successively dehydrated (PksH) and decarboxylated (PksI) as in the curacin case. It is probable that MupI and K along with MupH HMG-CoA synthase function in a similar manner to catalyze incorporation of an acetate at C-3, with the subsequent dehydration and decarboxylation required to generate the C-15 methyl group, in a similar fashion to the model for PA biosynthesis reported previously (10) (Fig. 2C). Our own recent studies have shown that in vivo mutation of mupH results in the production of a novel metabolite mupirocin H whose structure can be rationallyized as being formed by release of the polyketide substrate of MupH (31).

Operon organization and a requirement for cotranslation from the same mRNA have also been proposed to be important in other systems to ensure efficient translation and biological activity of proximal genes. For example assembly of the large multimeric E. coli F, F, ATPase enzyme is thought to be via a cis-assembly pathway (32). Similar to mutations of mupE and mupI, mutations of uncG and uncE were only complemented poorly by plasmids expressing just uncG or uncE alone, but could be complemented by a plasmid carrying all or several of the ATPase structural genes, respectively (33, 34). Brusilow proposes that co-translation of certain unc genes may therefore be required for their correct folding and insertion into the cell membrane. Likewise the mycobacterial plasmid pAL500 replication protein repB requires translational coupling to repA for increased efficiency of translation and protein folding (35). Binding of RepB to the origin of replication is only maximal when its expression is coupled to repA (36). It is proposed that ribosome-tethered chaperones dissociate slowly such that a ribosome reinitiating at a translationally coupled downstream gene is primed for folding and thus the downstream product is folded more efficiently than if independently translated. Protein association may also function to stabilize proteins. For example aspartate carbamoyltransferase of the extreme thermophile Thermus Z05 is inherently unstable unless associated with dihydroorotase, the next enzyme in the carbamoylation pathway (37).

Our initial studies on the mup cluster suggested that two ORFs mupF and mupP were transcribed counter to the rest of the cluster (10). The data presented here show that actually mupP runs in the same direction as the majority of the cluster, whereas we confirm that indeed mupF is transcribed in the reverse orientation. Furthermore without being under control of the tac promoter the reverse mupF ORF plus its upstream region is capable of complementing 10586ΔmupF. mupF must therefore be under the control of its own promoter. The upstream region of mupF contains reasonable −35 (TAGTCA) and −10 (TATACC) promoter sequences and a strong lux box candidate (ACCTATAAGACCTTGTAGTA) based on comparison to a lux box consensus from previously identified lux boxes (38). 10586ΔmupC was also complemented by mupC and the upstream mupF region without the control of the tac promoter. mupC must therefore also have its own promoter. Upstream of mupC there is an inverted repeat, which may be a putative lux box (CGGGTTAGGCGGGCTAAAGGA). Mutational analysis is underway to determine the importance of these sequences.

The fact that a point mutation in the active site of MacpC can be complemented by mupC expressed from the tac promoter in trans suggests that the in-frame deletion in mupC, which could not be complemented, removes sequences that are needed for a second function. We considered the possibility that the deletion in mupC removes sequences required for mupF transcription, but if this were the case then the presence of mupC in trans to the mupC deletion should restore the phenotype to that of a mupF knock-out and this does not seem to be the case. This suggests that there may be some other function encoded in this region such as a small RNA or additional polypeptide that remains to be discovered.

Our approach of disrupting tailoring ORFs and subsequent generation of novel shunt products has also proved useful in other polyketide systems to determine the role of post-PKS enzymes. For example mutagenesis of rifOrf5 in the rifamycin B biosynthetic pathway generated an early intermediate rifamycin W. This revealed that the Rif-Orf5 cytochrome P450 monoxygenase was responsible for conversion of the C-12,29 olefinic bond in rifamycin W into the ketal moiety of rifamycin B (39). LnDM2, a putative oxygenase-reductase enzyme of the landomycin E biosynthetic cluster, was inactivated in Streptomyces globisporus. Novel shunt products provided evidence for involvement of LnDM2 in early post-PKS tailoring steps, in particular C-6 hydroxylation and reduction (40). Directed inactivation of aviG4 and aviH of the avilamycin pathway also generated two new antibiotics confirming roles as methyltransferase and halogenase, respectively (41). Disruption of inter alia
Mupirocin Biosynthesis Tailoring Region

*ambM*, encoding a discrete C-methyl-transferase in the ambruticin gene cluster in *Sorangium cellulosum*, results in the production of 15-demethylambruticins (42).

Deletion mutagenesis of *mupC* results in a metabolite that could be rationalised as arising from a failure to reduce both a C-8,9 double bond and a C-7 ketone during polyketide chain assembly, whereas mutagenesis of *mupF* would result in failure to reduce a C-7 ketone only. Indeed, sequence comparisons are consistent with MupF acting as a ketoreductase, whereas MupC demonstrates homology to a dienoyl CoA reductase and other oxidoreductases, which would be consistent with it acting after a dehydration step by reduction of the C-8,9 double bond in the module 3 tetraketide intermediate (see supplemental data, Fig. S1). However, this contradicts our previous conclusion about the action of MupO/U/V/MacpE, which also seemed to be implicated in reduction of the C-8,9 double bond (14). To help resolve this we created double mutants of each of these with *mupC* and the fact that they also accumulated PA-B indicated that MupC works after MupO/V. Because MupU is a putative acyl CoA synthase, it is reasonable to propose that it may load an intermediate onto MacpE, so conceivably MupC may act on an intermediate also attached to MacpE. We previously proposed that MupO/V/U/MacpE work late in the biosynthetic scheme after the esterification of the MA precursor with 9HN but prior to pyran ring formation. However, the structure of mupirocin C suggests the possibility that MupO/V/U/MacpE and MupC may actually work during the elongation of the MA backbone, possibly between putative modules 4 and 5. Another possible explanation is that MupO/U/V/mAcpE and MupC act after MupW, and any partner proteins that it may work with, e.g., the putative dioxygenase ferredoxin subunit MupP. The pathway proposed in Fig. 6 is consistent with this latter proposal and with the observed metabolites isolated as a result of mutagenesis of *mupC* and *mupF*. According to this proposal, PA-B would be a direct intermediate on the pathway to PA-A. This is somewhat counterintuitive as most biosynthetic precedent would suggest that PA-B is produced by simple hydroxylation of PA-A. Thus we propose that the product of the MmpD module 4 is the tetraketide as shown in Fig. 2B but with an exo-double bond between C-8 and C-16 (PA numbering) rather than the conjugated dienoyl intermediate. This could be formed by double bond migration between the module 4 KS- and KR-mediated steps. Consistent with this, detailed analysis of the module 4 sequence downstream of the KS reveals a peptide sequence of ca. 470 amino acids, corresponding to a catalytic domain which was not previously assigned. Blast analysis does not reveal any obvious homologies, but it may be worth noting that this region between the KS and KR often houses a DH domain, which are notoriously difficult to assign. We tentatively suggest that this “domain” could be an isomerase responsible for the proposed double bond migration. Chain assembly would then proceed as previously proposed to produce the heptaketide 1 (Fig. 6). MupW catalyzed epoxidation of the C-8,16 double bond and attack of the C-5 hydroxyl group on the resulting epoxide would give rise to 2 that would rearrange to the trihydroxy-terahydropyran, which could be converted to PA-B after esterification with 9-HN and C-10,11 epoxidation, the timings of which remain to be established. Normal biosynthesis, however, would proceed by MupU mediated transfer to MacpE. Oxidation of the C-7 hydroxy to the ketone 3 catalyzed by the *mupO*-encoded cytochrome P450 would activate the molecule for MupV-catalyzed dehydration to give the fully conjugated dienoylketone 4. The C-8,9 olefin would be selectively reduced by MupC to give 5, which on further MupF catalyzed keto-reduction would give 6, which would be converted to PA-A as for PA-B. This sequence would be fully consistent with the production of small amounts of PA-B along with the major WT metabolite PA-A, presumably from the MupU-catalyzed transfer of 2 to MacpE not being completely efficient. Blocking this transfer would divert the full metabolic flow to PA-B production as observed. Conversely this suggests a point for manipulation to promote nearer a 100% production of PA-A. The mutations described here provide the basis for further double and triple knockouts that should prove highly informative.

Thus we present further evidence for the interactions that provide the logic underlying the organization of the *mup* cluster despite the absence of apparent colinearity between the gene order and the proposed biosynthetic pathway. First, some “tailoring” gene products must assemble with the products of adjacent genes into multiprotein complexes and could represent candidates that may eventually fuse to create new multifunctional proteins. Second, the existence of an internal bidirectional promoter region partly decouples the order of gene expression from the position in the cluster. Third, multiple tailoring genes appear to be involved in tetrahydropyran ring formation. Furthermore, if the tailoring region from *mupC* to *mupW* had been acquired by one (or more) integration events of a circular DNA molecule then it is possible that *mupC* and possibly *mupF* had at one time been more closely linked to *mupO, U, V, macpE*, and *mupW* with which we propose they work closely. Manipulation to rearrange the cluster to group-related functions together and possibly fuse co-functional polypeptides may improve the efficiency of PA-A production.

Acknowledgments—We thank An Phan for identification of the putative *mupF* lux box motif. DNA sequencing was performed by the IJF-funded Genomics Laboratory in the School of Biosciences (J16/F13209). ES-MS analysis was performed by Peter Ashton, School of Chemistry, University of Birmingham.
