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Biosynthesis of Mupirocin by *Pseudomonas fluorescens* NCIMB 10586 Involves Parallel Pathways

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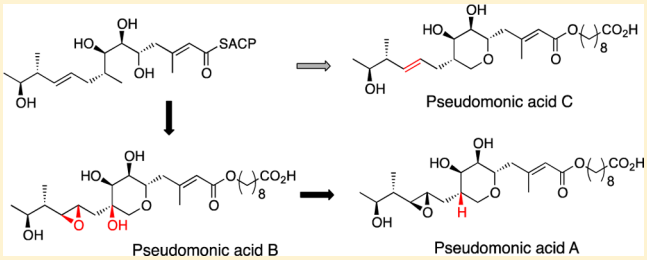
Supporting Information

ABSTRACT: Mupirocin, a clinically important antibiotic produced via a *trans*-AT Type I polyketide synthase (PKS) in *Pseudomonas fluorescens*, consists of a mixture of mainly pseudomonic acids A, B, and C. Detailed metabolic profiling of mutant strains produced by systematic inactivation of PKS and tailoring genes, along with re-feeding of isolated metabolites to mutant stains, has allowed the isolation of a large number of novel metabolites, identification of the 10,11-epoxidase, and full characterization of the mupirocin biosynthetic pathway, which proceeds via major (10,11-epoxide) and minor (10,11-alkene) parallel pathways.



INTRODUCTION

Mupirocin, a mixture of pseudomonic acids, produced by *Pseudomonas fluorescens* NCIMB 10586, is a clinically important antibiotic against Gram-positive bacteria, including methicillin-resistant *Staphylococcus aureus* (MRSA).[1,2] It inhibits bacterial isoleucyl-transfer RNA synthetase and is currently the standard treatment used worldwide for the topical control of MRSA.[3] The major component of mupirocin is pseudomonic acid A (PA-A, **1**, Figure 1), which accounts for ca. 90% of the mixture.[4] It was identified as one of the first of an extensive family of antibiotics produced by the "*trans*-AT" class of modular polyketide synthases (PKSs).[5] It consists of a C_{17} polyketide-derived substructure (monic acid) esterified by 9-hydroxynonanoic acid (9-HN). The other main components are pseudomonic acid B (PA-B, **2**, 8%), which has an additional hydroxyl group at C8,[6] and pseudomonic acid C (PA-C, **3**, <2%), which has a double bond in place of the epoxide group at C10−C11.[7] PA-D (**1**, 4′-5′-alkene) has also been reported as a very minor component. Simple biosynthetic logic would suggest that PA-A is formed by epoxidation of the 10,11-double bond in PA-C **3**, and PA-B **2**, by a further hydroxylation of PA-A at C8.

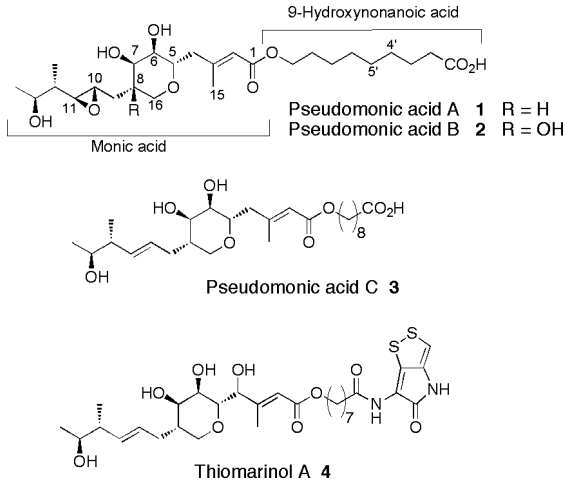


Figure 1. Major pseudomonic acids and thiomarinol A.

Novel pseudomonic acid analogues, the thiomarinols, e.g., thiomarinol A (**4**), also active against MRSA, have been isolated from marine organisms, e.g., *Pseudoalteromonas* sp. SANK 73390.[8] The thiomarinols are closely related to mupirocin,[9] being produced by a very similar biosynthetic gene cluster[10] with the addition of a non-ribosomal peptide synthase-encoded pyrrothine moiety attached via an amide to an 8-hydroxyoctanoic acid moiety. The 74-kb mupirocin biosynthetic gene cluster (Figure 2A) encodes six modular multifunctional proteins (Mmp's) involved in polyketide and fatty acid biosynthesis and 26 single enzymes originally thought to perform largely tailoring functions.[11] The first half of the cluster contains two large Type I multifunctional genes (*mmpA* and *mmpD*) plus associated *trans*-acyltransferases (*mmpC*), an iterative Type I fatty acid synthase (FAS) (*mmpB*), and two single open reading frames (ORFs) (*mupA* and *mupB*), while

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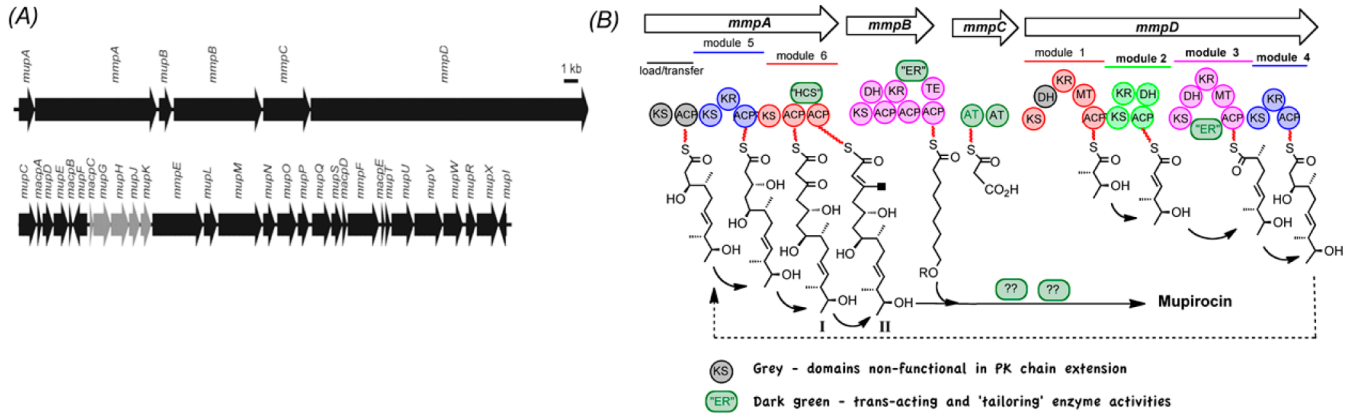

Figure 2. (A) Summary of the mupirocin biosynthetic gene cluster. The HCS cassette responsible for β-branching is shown in gray. (B) Formalized scheme for the biosynthesis of monic acid and its elaboration to muprocin. "Grey" domains are inactive or non-functional in chain elongation. Tailoring proteins acting *in trans* are indicated as elongated domains.

the second half contains 27 single ORFs (*mupC*–*X* and *macpA*–*E*) plus two smaller PKS-like genes (*mmpE* and *mmpF*). MmpD and MmpA together contain six modules for condensation and reduction of acetate-derived units, which with two methyl transferase domains could generate the backbone of a C_{17}-heptaketide monic acid precursor (**I** in Figure 2B). Module 6 of MmpA, the final module involved in monic acid biosynthesis, contains twin acyl carrier proteins (ACPs), each with a unique recognition motif specific[12] for association with the "β-hydroxymethylglutaryl-CoA synthase" (HCS) cassette (MupG, MupH, MupJ, MupK, and MacpC) responsible for the introduction of the 15-methyl β-branch (■ in **II** in Figure 2B).[13] 9-HN is likely formed from a 3-hydroxypropionate starter unit extended by three malonate condensations by MmpB functioning as an iterative FAS with additional enoyl reductase (ER) activity, perhaps provided by MupE and/or MupD.[14] The resulting backbone would need further modifications to produce the major metabolite PA-A.

In-frame deletions demonstrated that all of the proteins are required for mupirocin production.[14] However, many uncertainties remain, particularly in the timing of 10,11-epoxidation, tetrahydropyran (THP) ring formation, 6-hydroxylation, fatty acid chain extension, and the precise roles of, *inter alia*, the *mupW* (dioxygenase), *mupC* (oxidoreductase), *mupF* (ketoreductase), *mupU* (acyl CoA synthase), *mupO* (P_{450}), *mupV* (oxidoreductase), *macpE* (acyl carrier protein), and *mmpE* (ketosynthase/oxidoreductase) gene products.

Previous work on mutant strains containing single deletions of these genes led to the isolation of several novel PAs, including mupirocin W1 (**5**),[15] mupirocin C2 (**16**),[14] and mupirocins F1 (**18a**)[16] (previously named mupricins W, C, and F) from Δ*mupW*, Δ*mupC*, and Δ*mupF* strains, respectively (Figures 3 and 4 below). The tetrahydrofuran-containing metabolites **5** and **16** are likely rearrangement products formed by attack of a 7-hydroxyl group onto C-10 of the labile epoxide. Deletions of *mupV*, *mupO*, *mupU*, and *macpE* all resulted in a switch to production of PA-B **2** only,[17] and the corresponding double mutants with addition of a Δ*mupC* mutation gave the same result, suggesting that *mupV*, *mupO*, *mupU*, and *macpE* all act before *mupC* and, by implication, *mupF*. This led to the conclusion either that PA-B **2** is produced as a result of a branch in the pathway[17] or that it could be a precursor leading to PA-A **1**, as had been proposed but not proven by Mantle et al.[18] The 10,11-epoxide in PA-A **1** makes it susceptible to intramolecular attack by the 7-OH outside a narrow pH range, which limits its clinical utility.[2] PA-C **3**, lacking the epoxide, is similarly active to PA-A **1**, but it is much more stable. Thus, a desirable goal in terms of obtaining a clinically more useful antibiotic would be to knock out the epoxidase activity and channel production entirely to PA-C **3**. Further correlation of tailoring genes with catalytic function and resulting chemical modification was made difficult due to the so-called "leaky hosepipe" mechanism,[19] in which mutations of the HCS cassette and many others all produce an essentially identical phenotype in which pseudomonic acid biosynthesis is blocked and two truncated metabolites, mupiric acid (**13**) and mupirocin H (**14**) (Figure 3), are isolated. (Mupirocin H is proposed[19] to result from a retro-aldol cleavage of intermediate **23** in Scheme 1, below.) This phenotype was attributed to spontaneous release of these two compounds at chemically labile points, as a result of impeding metabolic flux along the assembly pathway by any mutation which interferes with formation of monic acid or its subsequent esterification by the MmpB-derived FAS.

We now report detailed investigations on mupirocin biosynthesis involving single and double gene knockouts, leading to new insights into this complex pathway, including association of MmpE with introduction of the 10,11-epoxide. Single mutants as well as the wild-type (WT) strain were grown on a modified L-medium, which crucially allowed more facile isolation of minor metabolites not previously observed in these strains. Similarly, a series of double mutants were analyzed under the same conditions, and intermediates isolated in these experiments were re-fed to mutant strains. As a result of these studies, definitive evidence has been obtained for the biosynthetic relationships among previously reported and novel pseudomonic acid metabolites, showing the presence of two parallel pathways, one major and one minor (see Scheme 1), and showing that epoxidation, THP ring formation, and 6-hydroxylation are necessary for efficient downstream metabolic processing.

■ RESULTS AND DISCUSSION

Analysis of WT and Mutant Strains. We first investigated metabolite production by the WT strain of *P. fluorescens* NCIMB 10586 cultivated on the modified L-medium to determine if any previously undetected minor metabolites were

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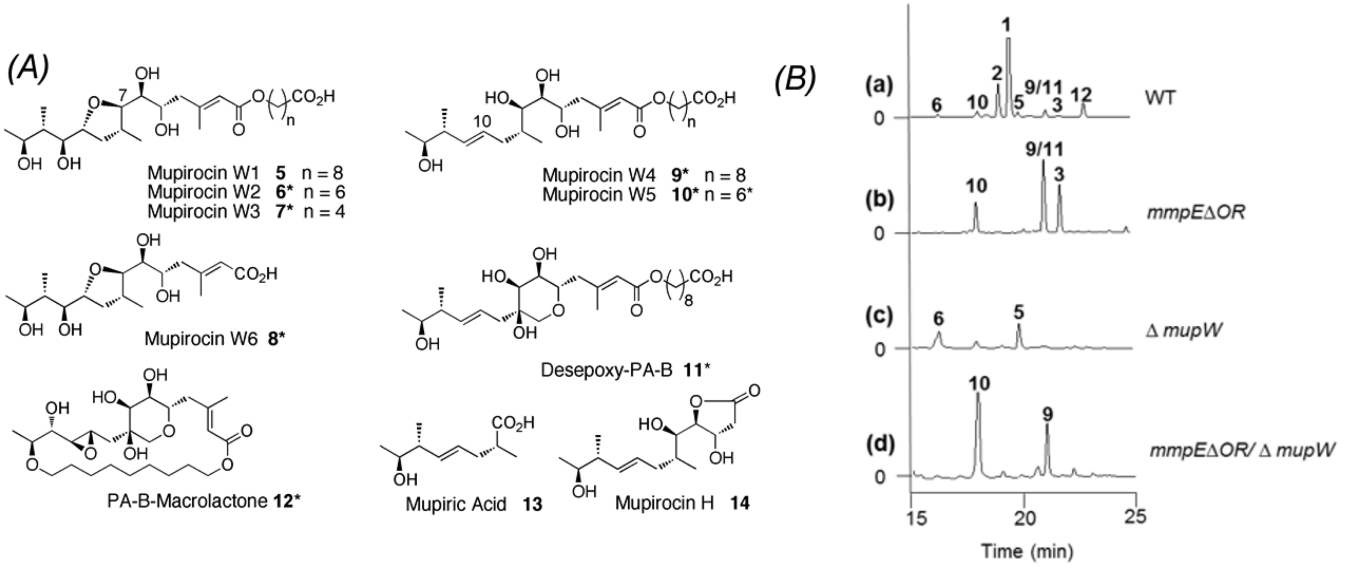

Figure 3. (A) Known and novel (*) metabolites isolated from extracts of WT and selected mutant strains of *P. fluorescens*. WT also contains PA-A and PA-B as the major products and a trace amount of PA-C. Metabolites in left column are epoxide derived, and those in the right column are 10,11-alkenes. Some compounds have been renamed to take account of new metabolites isolated. (B) HPLC traces of extracts of (a) WT, (b) *mmpEΔOR*, (c) Δ*mupW*, and (d) *mmpEΔOR*/Δ*mupW* strains.

Table 1. Pseudomonic Acids Isolated from WT and Mutant Strains

	WT	*mmpEΔOR*	Δ*mupW*	*mmpEΔOR*/Δ*mupW*	Δ*mupC*	*mmpEΔOR*/Δ*mupC*	Δ*mupF*	*mmpEΔOR*/Δ*mupF*	Δ*mupW/O*	Δ*KR6*
PA-A **1**	47									
PA-B **2**	3.8				7.6		7.1			
PA-C **3**[b]	0.2	2.6								
mupirocin W1 **5**	0.5		0.8		0.8		0.9		2.0	
mupirocin W2 **6**[a]	0.3		0.9		1.3		1.3		2.2	
mupirocin W3 **7**[a]									<0.5	
mupirocin W6 **8**[a]									<1.0	
mupirocin W4 **9**[a]	0.4	1.1		1.3	0.8	0.8	0.8	1.1		
mupirocin W5 **10**[a]	0.4	2.4		1.6	1.1	2.1	1.4	2.3		
10,11-deoxy-PA-B **11**[a,b]	0.3	2.3			0.7	1.8	1.1	1.7		
PA-B macrolactone **12**[a]	2.1				1.3		1.5			
mupirocin C1 **15**[a]					8.4		8.1			
mupirocin C2 **16**[a]					7.8					
desepoxy-mupirocin C1 **17**[a,b]						0.5		0.4		
mupirocin F1 **18**							8.4			
mupirocin F2 **19**[a]							8.1			
desepoxy-mupirocin F1 **20**[a,b]								1.3		
7-keto-mupirocin W4 **21**[a]										1.2
7-keto-mupirocin W5 **22**[a]										<1.0

[a]New PAs reported for the first time in this study. [b]PAs derived from minor pathway. Values are given in mg L^{-1}.

present (see Supporting Information (SI) for details). PA-A **1** (ca. 50 mg L^{-1}), PA-B **2**, and PA-C **3** were isolated (Figure 3B(a) and Table 1). Further detailed analysis of minor components resulted in the isolation of mupirocin W1 **5**[15] and its analogue mupirocin W2 **6** containing a shorter (C_7) fatty acid side chain, as well as four new mupirocin metabolites, of which one, a macrolactonic derivative **12** of PA-B, contained the 10,11-epoxide. The other three metabolites are mupirocin W4 **9** and mupirocin W5 **10**, each with a 10,11-alkene and hence lacking the tetrahydrofuran ring formed via the labile 10,11-epoxide, and 10,11-desepoxy-PA-B **11** (Figure 3A). These were isolated and purified, and their structures were determined by full NMR analysis and HR-ESI-MS (see SI for details). These metabolites form two structurally distinct groups. The first group contains the 10,11-epoxide (**1**, **2**, and **12**) or rearrangement products (**5** and **6**) derived from it, while the second group lacks the epoxide and contains a 10,11-alkene (**3**, **9**, **10**, and **11**). This suggests that the first group of PAs (**1**, **2**, **5**, **6**, and **12**), which account for >97% of total PAs in the WT strain (Table 1), are intermediates, shunt, or final natural products generated from the same dominant biosynthetic pathway, while a minor pathway involving intermediates lacking the 10,11-epoxide is responsible for the second group of PAs, accounting for <3% of the total yield of PAs.

Previous studies[14] failed to identify a monofunctional gene encoding a 10,11-epoxidase. We therefore considered domains of multifunctional proteins. One such candidate is the bifunctional MmpE, which has a putative *N*-terminal KS and

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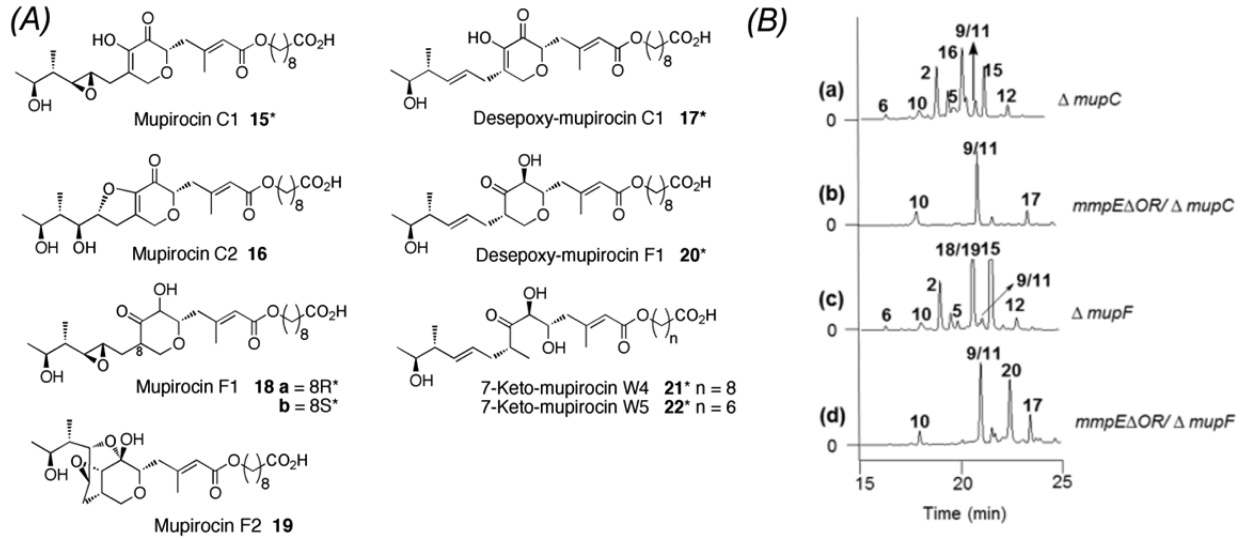

Figure 4. (A) Known and novel (*) metabolites isolated from extracts of selected mutant strains of *P. fluorescens*. Those in the left column are epoxide derived, and those in the right column are 10,11-alkenes. Some compounds have been renamed to take account of new metabolites isolated. (B) HPLC traces of extracts of (a) Δ*mupC*, (b) *mmpE*Δ*OR*/Δ*mupC*, (c) Δ*mupF*, and (d) *mmpE*Δ*OR*/Δ*mupF*.

a putative C-terminal oxidoreductase (MmpEOR). Deletion (aa789 to aa1173) of the predicted MmpEOR domain and HPLC analysis (Figure 3B(b)) indicated that production of PAs with a 10,11-epoxide or derived functionality are completely abolished, and only PAs with a 10,11-alkene (PA-C **3**, mupirocins W4 and W5 **9** and **10**, and desepoxy PA-B **11**) accumulate in the *mmpE*Δ*OR* mutant, consistent with *mmpEOR* encoding the 10,11-epoxidase. The titers of these 10,11-alkenes (2.6 to 1.1 mg L^{-1}) isolated from the *mmpE*Δ*OR* mutant, while somewhat higher than in WT (0.4 to 0.2 mg L^{-1}, Table 1), remain very low.

As indicated above, mutation of *mupW*[15] and also of *mupT* (which encode a putative dioxygenase and associated ferredoxin dioxygenase, respectively) produces mupirocin W1 **5**, lacking the THP ring, and so these genes appear to be responsible for the oxidative activation required for the formation of the ring.[15] In this study, we first re-fermented Δ*mupW*. HPLC (Figure 3B(c)) shows the presence of **5** and its 7-hydroxyheptanoate analogue mupirocin W2 **6** (each ca. 1 mg L^{-1}). The Δ*mupT* mutant gave identical results (data not shown). Interestingly, a double Δ*mupW*/Δ*mupO* mutant (originally constructed to test the relative timing of action of MupW and MupO) gave slightly higher titers of **5** and **6** and also allowed detection of smaller amounts of mupirocin W3 **7**, an analogue containing a further truncated 5-hydroxypentanoate side chain, and, significantly, mupirocin W6 **8**, the only monic acid analogue so far isolated lacking a fatty acid side chain. To explore the relationship between epoxidation and other tailoring steps, the double mutant *mmpE*Δ*OR*/Δ*mupW* was then examined. Production of **5** and **6** was abolished, and only their desoxy 10,11-alkene analogues, mupirocins W4 **9** and W5 **10** (each ca. 1.5 mg L^{-1}), accumulated (Figure 3B(d)). Although the actual substrates for MupW/T and MmpEOR remained to be established (see below), it is notable that MmpE (Figure 2) is positioned immediately after the "HCS cassette" responsible for the introduction of the *β*-branch 15-methyl.[13] Also, in the related thiomarinol gene cluster,[10] the tmlK and tmpE genes are fused (Figure S2), encoding a tri-functional protein, suggesting that these activities act together or in sequence—i.e., after the action of the final module of MmpA, and immediately before THP formation (see intermediate **25** in Scheme 1, below).

The Δ*mupC* mutant was also analyzed and showed the presence of three major, and various minor, metabolites (Figure 4B(a)). The three major products (80% total PAs, each ca. 8 mg L^{-1}, Table 1) were identified as the novel mupirocin C1 **15**, rearrangement product[14] mupirocin C2 **16** (presumably formed from **15**), and PA-B **2**. Both mupirocins C1 and C2 have C6 and C7 at the ketone oxidation level. The minor products (0.7−1.3 mg L^{-1}) were analogous to those seen in WT: mupirocin W1 **5**, W2 **6**, W4 **9**, W5 **10**, desepoxy-PA-B **11**, and macrolactone **12**.

The double mutant *mmpE*Δ*OR*/Δ*mupC* produced mainly the 10,11-alkene containing PAs **9**, **10**, and **11** (Figure 4B(b)) and a new product identified as desepoxy-mupirocin C1 **17**. MupC was identified as a putative dienoyl-CoA reductase,[11] suggesting that it acts here on the enol ketones **15** and **17**. A similar enone reductase activity has recently been reported for a reductase from a *Clostridium* sp. which shows high homology to dienoyl CoA reductases.[20]

When the Δ*mupF* strain was examined (Figure 4B(c)), the metabolite profile was almost identical to that from Δ*mupC*, with the addition of the previously reported[16] mupirocin F1 **18a** containing a 6-hydroxy, 7-keto moiety (along with 50% of its 8-epimer **18b**) and its epoxide-mediated rearrangement (see Scheme S1) product mupirocin F2 **19** as additional major (ca. 8 mg L^{-1}) metabolites. The minor metabolite yields were essentially identical to those in the Δ*mupC* mutant.

As with Δ*mupC*, formation of the double mutant *mmpE*Δ*OR*/Δ*mupF* simplified the metabolite profile (Figure 4B(d)), which was very similar to *mmpE*Δ*OR*/Δ*mupC*, apart from a new metabolite which was identified as desepoxy-mupirocin F1 **20**, the 10,11-alkene analogue of mupirocin F1 **18**. This and the results from the single mutant Δ*mupF* are consistent with MupF acting as a 7-ketoreductase with both hydroxyketones **18** and **20**.

Metabolites from double mutants Δ*mupW*/Δ*mupC* and Δ*mupW*/Δ*mupU* are identical to those of single mutant Δ*mupW* (Figure S3), and no other metabolites from the single mutants, Δ*mupC* or Δ*mupU*, are present: this confirms that

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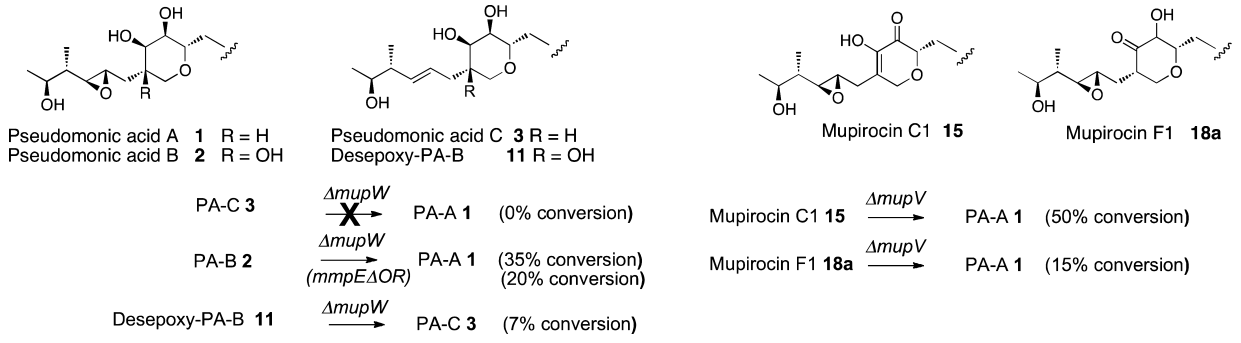
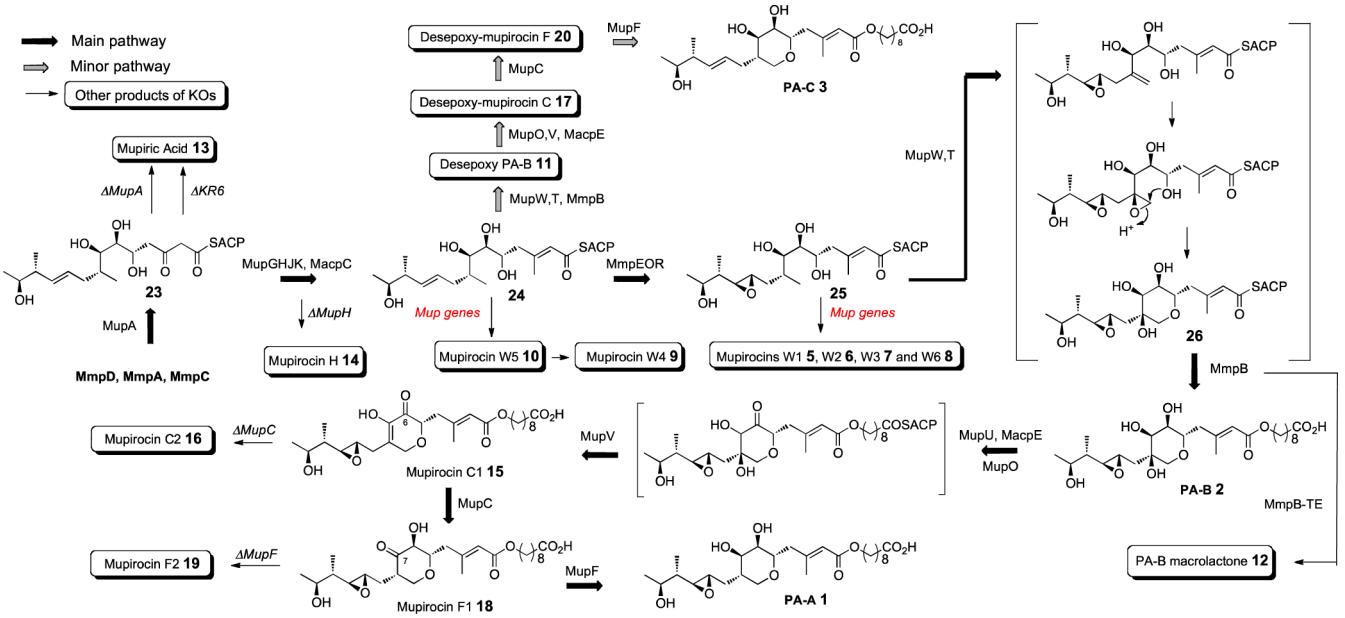


Figure 5. Feeding of isolated mupirocin metabolites to mutant strains of *P. fluorescens*.

Scheme 1. Biosynthetic Pathway to Mupirocin and Related Metabolites[a]



[a]Structures of intermediates on the main pathway are shown. For minor pathway and other compounds, see Figures 3 and 4.

MupW acts before MupO, MupU, MupV, and MupC. The Δ*mupC*/Δ*mupF* double mutant is identical to the single mutant Δ*mupC*, confirming that MupC acts before MupF. Interestingly, re-examination of the strain in which the KR domain (KR6) responsible for initial reduction at C7 during assembly has been deleted led to isolation of the previously reported[19] truncated product mupiric acid **13**, along with the 7-keto analogues **21** and **22** of mupirocin W4 and W5. Thus, a 7-hydroxyl group is clearly required for action of MmpEOR and MupW/T.

No compounds lacking a 6-OH have been detected. The gene responsible for 6-hydroxylation has not been definitively identified but must function before the HCS cassette, as evidenced by the isolation of mupirocin H **14** from mutation of any of the HCS cassette genes,[19] or either of the MmpA twin acyl carrier proteins.[12] Mutation of *mupA*,which encodes a putative FMNH-dependent oxygenase, again gives the shunt metabolite mupiric acid **13** as the only detectable product. Thus, 6-hydroxylation may be controlled by *mupA* and seems to be essential for further processing along the main pathway.

Chemical Complementation Experiments. To further clarify the later stages of the biosynthetic pathways and to identify the substrates for the later-acting enzymes, a series of feeds to small-scale cultures (25 mL) were performed. Metabolites were fed to the mutant strains immediately after inoculation, and the extracts were analyzed by HPLC-MS (Figures 5 and S4). We first fed PA-C **3** to Δ*mupW*, in which the *mmpE* gene is still intact and could potentially catalyze 10,11-epoxidation. After 60 h fermentation, mupirocin W1 **5** and W2 **6** were produced as normal, but no metabolism of PA-C **3** and no production of PA-A **1** was detected. This is consistent with PA-A **1** and PA-C **3** being formed from a common intermediate by parallel pathways that diverge at 10,11-epoxidation. To further confirm this, PA-B **2** was fed to mutants Δ*mupW* and *mmpE*Δ*OR*. PA-A **1** was produced in both cases with conversion rates of 35 and 20%, respectively (Figures 5 and S4a,d), consistent with PA-B **2** being an intermediate on the main pathway to PA-A **1** (Scheme 1), as originally proposed by Mantle.[18] On feeding the 10,11-alkene analogue **11** of PA-B to Δ*mupW*, PA-C **3** is produced (7% conversion, Figures 5 and S4b), but no PA-A **1**. Thus, neither PA-C **3** nor the 10,11-alkene analogue of PA-B **11** or intermediates derived from it can be the actual substrate for MmpEOR. Therefore, it seems that MmpEOR acts before MupW/T, and the parallel pathways must be active at the same time in the WT strain since both epoxidized and non-epoxidized intermediates (see **24** and **25**, Scheme 1) can be processed to generate analogous products.

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Mupirocins W4 **9** and W5 **10** were also fed to the *mupH* strain, in which biosynthesis is truncated at the *β*-methylation (15-methyl) stage.[19] No transformation of mupirocin W4 **9** was observed, but mupirocin W5 **10** (C_7 side chain) was elongated to mupirocin W4 **9** (C_9 side chain), indicating that these intermediates can be taken up and metabolized by the cells (Figure S4i). This suggests that once the fatty acid side-chain elongation is underway, THP ring formation is not possible, again consistent with both MmpEOR and MupW/T acting after polyketide assembly and before fatty acid side-chain elaboration.

Additions of the enol-ketone mupirocin C1 **15** to deletion strains of Δ*mupU*, Δ*mupV*, Δ*mupO*, and Δ*macpE* (which all produce PA-B **2** but not PA-A **1**) all (e.g., Figures 5 and S4e) gave good conversion to PA-A **1**, with most of the unincorporated substrate being transformed to its rearrangement product mupirocin C2 **16**. Similarly, PA-A **1** was obtained (Figures 5 and S4j) on feeding mupirocin F1 **18** to Δ*mupV*. These experiments confirm that MupC acts as an enone reductase[11] and MupF as a 7-ketoreductase.

CONCLUSIONS

We have elucidated details of the pathway to PA-B **2** and established its intermediacy in PA-A **1** biosynthesis. The efficient transformation of PA-B **2** to PA-A **1** and the switch to PA-B **2**-only production on mutation of any of *mupU* , *mupV*, *mupO*, *mupC*, *mupF*, and *macpE* suggest that modification of the pyran ring occurs after elaboration of the 9-hydroxynonanoic acid moiety on monic acid. This is consistent with the pathway shown in Scheme 1, in which *β*-keto ester **23**, produced by the modular proteins encoded by *mmpD*, *mmpA*, and *mmpC*, along with the putative oxidase mupA, is the substrate for the HCS cassette to give the acyclic monic acid thioester **24**. Thioester **24** is then epoxidised to give **25** and cyclized to **26**, which is the substrate for stepwise fatty acid elongation to PA-B **2**. This is then transferred (MupU) to MacpE, where it is oxidized (MupO), dehydrated (MupV), and stepwise reduced (MupC and MupF) to give PA-A **1**. It appears that release of PA-B from MmpB is dependent on the action of the terminal thioesterase (TE) domain. A *mmpB*Δ*TE* mutant gives only the mupiric acid/mupirocin H phenotype. This is further supported by the isolation of macrolactone **12** from WT and Δ*mupC* and Δ*mupF* strains. It is unlikely that macrocyclization could be spontaneous, which suggests that the TE domain can accept either the 13-OH or water to give intramolecular or intermolecular catalyzed release.

Isolation of, e.g., mupirocins **6**, **7**, **10**, and **22**, containing shorter fatty acid side chains, demonstrates that, while monic acids lacking the THP ring can act as substrates for MmpB-dependent 9HN elongation, it is inefficient, consistent with MupW/T acting before MmpB.

PA-C **3**, previously assumed to be a precursor to PA-A **1**, is formed by a minor parallel pathway. Several of the intermediates involved in these parallel pathways were isolated from a series of single and double mutants, and their involvement was demonstrated by their transformation to either PA-C **3** or PA-A **1** on re-feeding to mutant strains. We have identified the gene responsible for epoxidation. This occurs at the end of assembly of the monic acid moiety **24**, as indicated by lack of epoxidation on mupiric acid **13** and mupirocin H **14**, but epoxidation has taken place in formation of the acyclic monic acid, mupirocin W6 **8**. Thus, epoxidation can occur before MupW- and MupT-catalyzed THP ring formation, and ring formation is not essential for epoxidation. Nevertheless, since in all mutant strains and WT, non-epoxy metabolites appear in very low titers, epoxidation, like THP ring formation, seems to be important for further processing of intermediates by later-stage enzymes. Thus, our goal of engineering *P. fluorescens* to give high titers of only PA-C may be unachievable, and other means of achieving this are currently being explored.

ASSOCIATED CONTENT

Supporting Information

Complete description of materials and methods, and additional tables, figures, and a scheme, including full ^{1}H and ^{13}C NMR data for all new compounds.This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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