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Tandemly Duplicated Acyl Carrier Proteins, Which Increase Polyketide Antibiotic Production, Can Apparently Function Either in Parallel or in Series*

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Ayesha S. Rahman^{‡§}, Joanne Hothersall[‡], John Crosby[¶], 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

Polyketide biosynthesis involves the addition of subunits commonly derived from malonate or methylmalonate to a starter unit such as acetate. Type I polyketide synthases are multifunctional polypeptides that contain one or more modules, each of which normally contains all the enzymatic domains for a single round of extension and modification of the polyketide backbone. Acyl carrier proteins (ACP(s)) hold the extender unit to which the starter or growing chain is added. Normally there is one ACP for each ketosynthase module. However, there are an increasing number of known examples of tandemly repeated ACP domains, whose function is as yet unknown. For the doublet and triplet ACP domains in the biosynthetic pathway for the antibiotic mupirocin from *Pseudomonas fluorescens* NCIMB10586 we have inactivated ACP domains by in-frame deletion and amino acid substitution of the active site serine. By deletion analysis each individual ACP from a cluster can provide a basic but reduced activity for the pathway. In the doublet cluster, substitution analysis indicates that the pathway may follow two parallel routes, one via each of the ACPs, thus increasing overall pathway flow. In the triplet cluster, substitution in ACP5 blocked the pathway. Thus ACP5 appears to be arranged “in series” to ACP6 and ACP7. Thus although both the doublet and triplet clusters increase antibiotic production, the mechanisms by which they do this appear to be different and depend specifically on the biosynthetic stage involved. The function of some ACPs may be determined by their location in the protein rather than absolute enzymic activity.

Acyl carrier proteins (ACP(s))¹ are small (80–110 amino acids) acidic proteins or domains that are essential to a variety of metabolic pathways. The active site serine of the ACPs is joined to the 4'-phosphopantetheine prosthetic group by a phosphodiester linkage to form the holoenzyme ACP. Reaction intermediates are attached to the terminal thiol of the 4'-

phosphopantetheine arm that swings between enzymes to perform its function (1), allowing interaction with almost every other enzyme in the pathway. ACPs are involved in fatty acid synthesis in all living organisms. They can also act as acyl donors in the biosynthesis of lipids (2), can carry membrane-derived oligosaccharides (3), and can be involved in the activation of RTX toxins in Gram-negative bacteria (4). Specialized ACPs may also act as signal factors in rhizobial nodulation (5) as well as substrates and carriers in the synthesis of lipoteichoic acid (6) and polyketide antibiotics (7).

Polyketides are assembled by successive condensations of small carboxylic acid derivatives catalyzed by enzymes called polyketide synthases (PKS), which are homologous to fatty acid synthases (8). ACPs thioesterified at the phosphopantetheine cofactor with the chain extender unit are used to generate nucleophiles to be acted upon by PKSs during condensation reactions. There are basically three types of polyketide synthases, the multifunctional Type I modular systems, the discrete Type II iterative synthases (9), and the Type III chalcone synthases, which consist of a single homodimeric enzyme working iteratively independent of ACPs (10). Programming exhibited by PKS can be modular, where each enzyme works just once in the biosynthetic process, and iterative, where the enzymes work many times. The iterative Type I system of fungi has a single large polypeptide with a set of active site domains as in Type I modular systems but working iteratively to produce the polyketide. Modular systems like erythromycin have a single ACP/module, one for each condensation cycle the growing polyketide chain undergoes before the completed chain is released, so that each ACP domain is used only once in the course of each biosynthetic cycle (11). Most of the Type II systems in bacteria contain a single ACP that works iteratively with other PKS enzymes performing the dual function of receiving the chain extender unit before condensation and of holding the growing polyketide chain after each condensation to produce the polyketide (12, 13).

Despite the above generalizations about Type I PKS systems there are an increasing number of examples with tandem clusters of ACPs, for example in the naphthopyrone and sterigmatocystin PKS of *Aspergillus nidulans* (14, 15) and the albicidin biosynthetic cluster of *Xanthomonas albilineans* (16), although the role of such duplication is not known. Gene duplication is commonly proposed as a way that new functions arise, the second stage being either silencing of one of the gene copies by deleterious/degenerative mutations yielding pseudogenes or development of a novel function by one of the gene copies. Recently, however, the idea of subfunctionalization has evolved, which proposes the idea of partitioning the tasks of the ancestral gene between the pair (17). Elucidation of the reason

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§ Supported by a studentship from the Darwin Trust of Edinburgh.

¶ To whom correspondence should be addressed. Tel.: 44-121-414-5903; Fax: 44-121-414-5925; E-mail: c.m.thomas@bham.ac.uk.

¹ The abbreviations used are: ACP, acyl carrier protein; TE, thioesterase; KS, ketosynthase; PKS, polyketide synthase; Mmp, mupirocin multifunctional polypeptide; HPLC, high performance liquid chromatography.

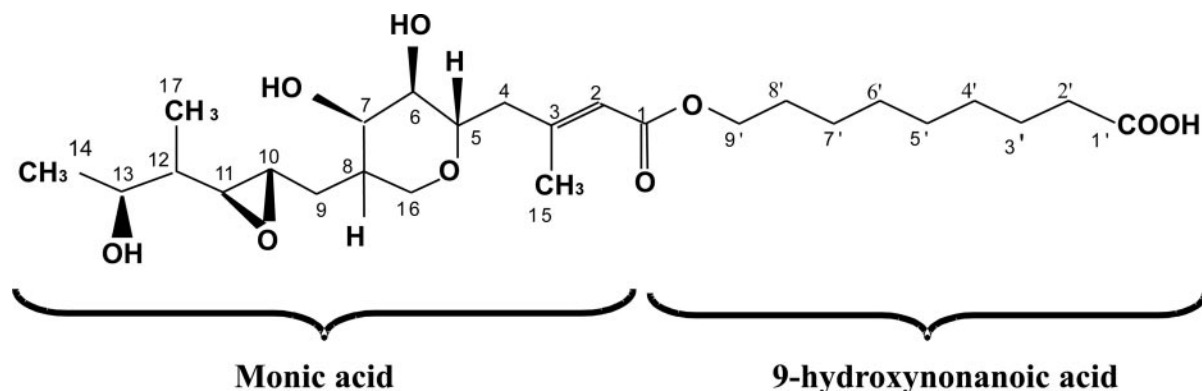


FIG. 1. The structure of mupirocin consisting of monic acid and 9-hydroxynonanoic acid. The form shown is pseudomonic acid A, which constitutes about 90% of mupirocin. PA-B (~5% of mupirocin) differs by having a hydroxyl at C-8; PA-C (~2%) lacks the epoxide ring and has a double bond between C-10 and C-11; PA-D (also ~2%) differs in 9-hydroxynonanoic acid having an unsaturated double bond at position 4'-5'.

for ACP domain duplication may not only shed light on the process of polyketide biosynthesis but may also provide more general insights.

Two examples of tandemly repeated ACP clusters are found in the mupirocin biosynthetic gene cluster from *Pseudomonas fluorescens*, a Gram-negative soil-borne bacterium (18). Mupirocin or pseudomonic acid (PA) is an antibiotic that competitively inhibits isoleucyl-tRNA synthetase and prevents the incorporation of isoleucine into growing polypeptide chains (19). It is used topically in the treatment of skin and burn wound infections or applied intranasally to control hospital outbreaks of methicillin-resistant *Staphylococcus aureus* (20, 21). Mupirocin is formed by the esterification of 9-hydroxynonanoic acid and monic acid (22) (see Fig. 1). Oxygen labeling experiments confirmed that this ester linkage is formed between separate C₁₇ and C₉ moieties (23). The ~74-kb mupirocin biosynthetic cluster involved in mupirocin production was identified by transposon mutagenesis and reverse genetics (24). DNA sequencing (18) revealing six long open reading frames typical of Type I PKS proteins, which we named mupirocin multifunctional polypeptides (MmpA–MmpF) as well as 27 genes (*mupA–mupZ* and *macpA–macpE*) encoding discrete proteins, some of which are similar to Type II PKSs. Quorum sensing regulates the expression of genes in the mupirocin biosynthetic cluster, which was repressed during the exponential phase and maximal on entry into stationary phase (25). Monic acid synthesis is predicted to involve six condensation reactions catalyzed by six modules the last of which contains a pair of tandem ACPs, whereas the region currently hypothesized to be linked to 9-hydroxynonanoic acid production has a tandem cluster of three ACPs (18). Other characteristics unique to the mupirocin cluster are the absence of a loading domain, the apparent absence of AT domains apart from AT1 and AT2 of MmpIII, the unusual position of the thioesterase domain, and the presence of 16 acyl carrier domains (18). In this paper we establish for the first time a phenotype for the tandem repetition of Type I ACPs. Although the results show that in both cases the pathway can continue if just one of the ACPs is present, the results are not consistent with all ACPs being equal.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Media Used—*P. fluorescens* NCIMB10586 (24) the producer of pseudomonic acid was used as the wild type strain for creating mutants. *Escherichia coli* DH5 α (26) was used for plasmid transformation and propagation, and strain S17-1 (27) was used to mobilize suicide and expression plasmids into the wild type *P. fluorescens* NCIMB10586. *Bacillus subtilis* 1064 was used as the sensitive organism in the bioassays to detect the antibacterial activity of mupirocin produced by wild type as well as mutants if any were produced (24). *P. fluorescens* NCIMB10586 was grown at 30 °C in L-broth/agar containing 50 μ g/ml ampicillin or 50 μ g/ml kanamycin.

Mupirocin production medium containing 2.3 g/liter yeast extract, glucose 1.1 g/liter, Na₂HPO₄ 2.6 g/liter, KH₂PO₄ 2.4 g/liter, and (NH₂)₂SO₄ 5.0/liter was used in preparing cultures for HPLC.

DNA Isolation and Manipulation—Plasmid DNA was extracted using the alkaline SDS method (28) or by Wizard Plus SV Minipreps DNA Purification Systems (Promega). The digestion of DNA was carried out with appropriate restriction enzymes (MBI Fermentas). The PCR products were cloned into vectors using T4 DNA ligase (29).

Construction of Deletion Mutants Using Suicide Vector Strategy—The suicide vector pAKE604 containing the Amp^R, Kan^R, *lacZ* α , *sacB* as selectable markers with an *oriT* was used to construct the deletion mutants (18). The general procedure involves designing two sets of primers with 5'-restriction sites to amplify 500 bp-flanking regions on either side of the gene to be deleted by PCR and ligating them. The ~1-kb insert was introduced into pAKE604 and *E. coli* S17-1 was transformed to mobilize the plasmid into *P. fluorescens*. Point mutations in mACP3, mACP4, mACP5, and mACP7 were constructed by replacing the active site codon TCG encoding serine in the wild type by GCC encoding alanine in one of the primers, and a restriction site was introduced by altering the third codon without changing the amino acid it encodes. The suicide derivatives of pAKE604 constructed in this way were transferred from *E. coli* S17-1 into wild type *P. fluorescens* NCIMB10586. Both of the strains were mixed and filtered through 0.45 μ m sterile Millipore filters, which were then placed on L-agar plates to allow conjugation. *P. fluorescens* with the suicide plasmid integrated into the chromosome was then selected on M9 minimal medium with kanamycin. Medium containing 5% (w/v) sucrose was subsequently used to select strains in which the suicide plasmid had been excised from the chromosome. Screening for sensitivity to kanamycin identified bacteria where excision of the suicide plasmid had occurred.

DNA Sequencing—Automated sequencing of the plasmid DNA was carried out by ABI Prism BigDye V3 Terminator ready reaction kit (PE-ABI), which has *ampliTaq* DNA polymerase, dye terminators, deoxynucleoside triphosphates, magnesium chloride, and buffer premixed into a single tube of Ready Reaction Mix and is based on the chain termination method of Sanger *et al.* (30). The sequencing reaction was performed on ABI 3700 DNA analyzer (Genomics facility, University of Birmingham).

Bioassay—Single colonies of *P. fluorescens* NCIMB10586 wild type/mutants were inoculated into L-broth containing ampicillin and incubated overnight at 30 °C. After normalizing the optical density measured at 600 nm, cultures were spotted onto L-agar plates and incubated at room temperature for 18–24 h. These plates were overlaid with a mixture of L-agar, *B. subtilis* culture (strain 1064 grown at 37 °C to late log phase), and 2,3,5-triphenyl tetrazolium chloride (0.025%) as an indicator and incubated at 37 °C overnight. The zone of clearance around the spot culture was taken as an estimate of the amount of mupirocin produced.

Culture Conditions for Isolation of Compounds—To obtain the seed culture a loop full of wild type and mutant cultures of *P. fluorescens* NCIMB10586 were inoculated into 25 ml of L-broth in 250-ml conical flasks and were shaken at 25 °C for 24 h. Five ml of the seed culture was inoculated into 25 ml of mupirocin production media and shaken at 22 °C for 20–60 h. The cultures were collected at different times, and bacteria were removed by centrifugation at 25 °C. The supernatant was used for HPLC analysis. Before injection the samples were filtered through 0.2 μ m Acrodisc Nylon filters (Gelman Laboratory-Fischer).

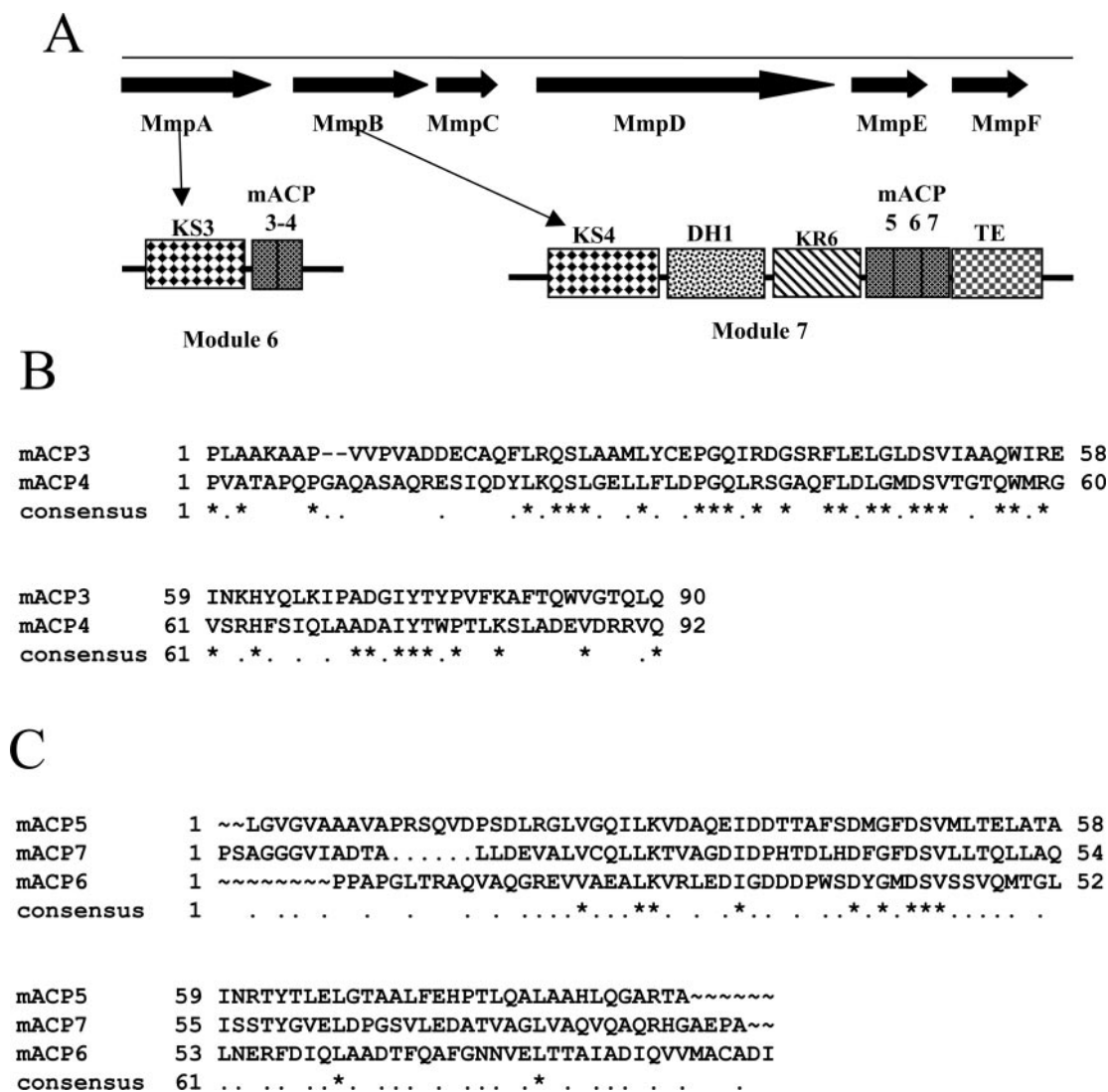


FIG. 2. *A*, organization of the mupirocin multifunctional polypeptides and the position of the doublet and triplet ACPs in the Mmps. The *mmps* are interspersed by regions containing discreet Type II proteins not shown. *KR*, ketoreductase; *DH*, dehydratase. For more details of the cluster refer to El-Sayed *et al.* (18). *B*, the sequence alignments of the doublet ACPs and triplet ACPs were performed using the program ClustalW.

Analysis of Compounds by Reverse Phase High Performance Liquid Chromatography—HPLC analysis was carried out to compare product profiles of the wild type *P. fluorescens* NCIMB10586 and mutant derivatives. HPLC (Gilson) with UV detector was used at a sensitivity of 0.002 absorbance units full scale and a wavelength of 233 nm. The Discovery^R C-18 silica column (Supelco) used had a pore size of 5 μ m. The solvents used were HPLC-grade water (Fischer) and HPLC-grade 100% acetonitrile (Fischer). Trifluoroacetic acid was added to adjust the pH of the mobile phase. A 70% acetonitrile gradient was used to elute mupirocin and the intermediates at a flow rate of 1 ml/min.

RESULTS

Amino Acid Sequence Similarities between Doublet and Triplet ACPs—For all the doublet and triplet ACPs, the active site serine residue lies in the highly conserved DSV motif characteristic of acyl carrier proteins (Fig. 2). The modular mACPs contain almost equal numbers (~90) of amino acids, but show a high degree of sequence divergence (19–42% sequence identity), ruling out very recent gene duplication. Pairwise alignments (Fig. 2B) showed that mACP3 and mACP4 are more related to each other (40% identical) than to mACP5/6/7. Within the triplet, mACP5 and mACP7 (42% identical) are more closely related, but mACP5, mACP6, and mACP7 as a group are more closely related to each other than to mACP3/4. The similarity/identity is consistent with the mean similarity

observed between tandemly duplicated genes of prokaryotes and eukaryotes (25–30%) (31). The tandem ACPs can, therefore, be described as paralogous genes and might have diverged in sequence after ancient tandem duplication events. We were not able to identify any heterologous ACPs more closely related, which might have supported the idea of recent acquisition from an alternative source. Thus the closest identified relative of mACP3 is mACP4 and therefore they might have originated by gene duplication. A similar situation is found with mACP5 and mACP7, whereas mACP6, which seems to have originated from a different ancestor, might have been inserted between the two other mACPs of the triplet by a random recombination process. Alternatively it could be that mACP6 and mACP5/7 are the result of an initial duplication with mACP5/7 resulting from a later duplication.

Construction of Mutants of the Putative Acyl Carrier Proteins—The mACPs in this study are part of multifunctional megaproteins and so it was important to achieve inactivation without affecting the other functions in the protein. Initial analysis was therefore by in-frame deletions. These deletions were constructed in the suicide plasmid pAKE604 individually and introduced into *P. fluorescens* NCIMB10586 as described under "Experimental Procedures."

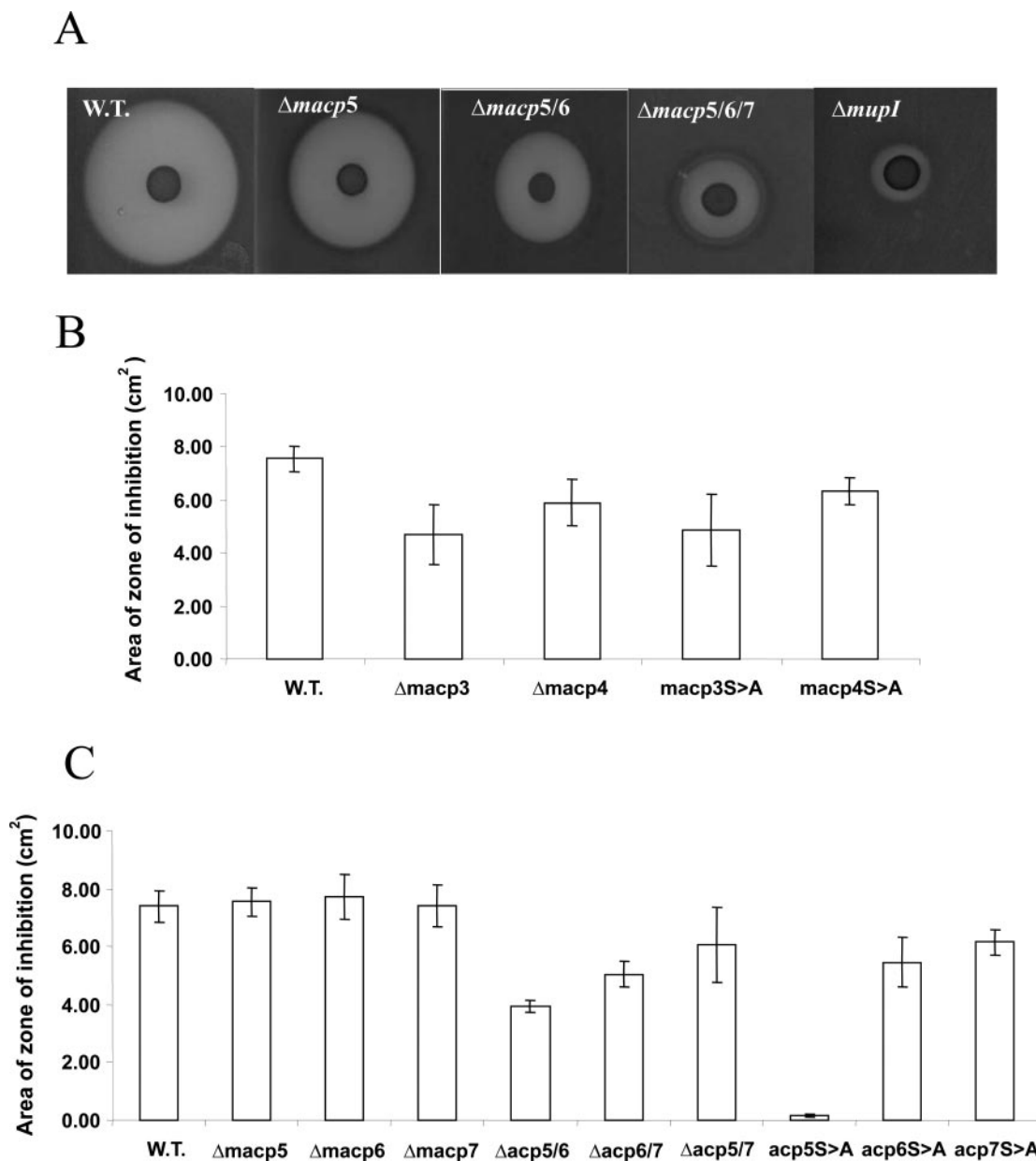


FIG. 3. A, bioassay for mupirocin production illustrated by zones showing the reduction in activity with successive deletion of genes from the triplet ACP cluster. *B. subtilis* was used as the test organism. The zone of clearance around the central spot culture shows the antibacterial activity. The areas of these zones were measured for all the mutants described in this paper with the results shown in B and C.

Because the *mmpA* and *mmpB* genes appear to be part of a larger polycistronic transcriptional unit, all deletion mutations were constructed in-frame to remove only specific runs of amino acids and avoid polar effects on the transcription of the downstream genes. Within the Mmp proteins the spacer regions between domains are usually 100 residues long, but mACP3 and mACP4 are separated by 12 amino acids, whereas mACP5 and mACP6 and mACP6 and mACP7 are separated by only 3 amino acids in both cases. The double and triple mutants were constructed by deleting completely the mACP doublet and mACP triplet including the spacer regions between these domains, respectively, from the mupirocin cluster. The single/individual deletions were made in such a manner that the spacer regions between the individual domains of an Mmp were retained to allow correct three-dimensional folding and orientation of domains of the polypeptide. The most complicated construction was that removing mACP7 from the mutant lacking mACP5, so as to leave mACP6 intact. Point mutations were also constructed by replacing the active site serine of tandem

mACPs with an alanine, so as not to alter the natural folding of the polypeptide. The mutations were checked by PCR amplification and confirmed by sequencing.

Qualitative Analysis of Mutants by Bioassay—To compare the antibacterial activity possessed by the wild type and mutant strains, a bioassay was performed using *B. subtilis* 1064 as the test organism. Indicator plates containing 2,3,5-triphenyl tetrazolium chloride, which acts as a terminal electron acceptor and turns red when bacteria are actively growing, were used to estimate the zone of inhibition produced around the spot culture of wild type *P. fluorescens* (Fig. 3A). By contrast, the $\Delta macp3/4$ double domain deletion mutant phenotype was indistinguishable from that of the *mupI* mutant (lacking the quorum regulation autoinducer needed to switch on *mup* expression in stationary phase) that is used as a negative control to demonstrate the loss of antibiotic production. The zones of inhibition of the individual $\Delta macp3$, $\Delta macp4$, $\Delta macp5$, $\Delta macp6$, and $\Delta macp7$ mutants were comparable with or only slightly smaller than that of wild type *P. fluorescens*, whereas

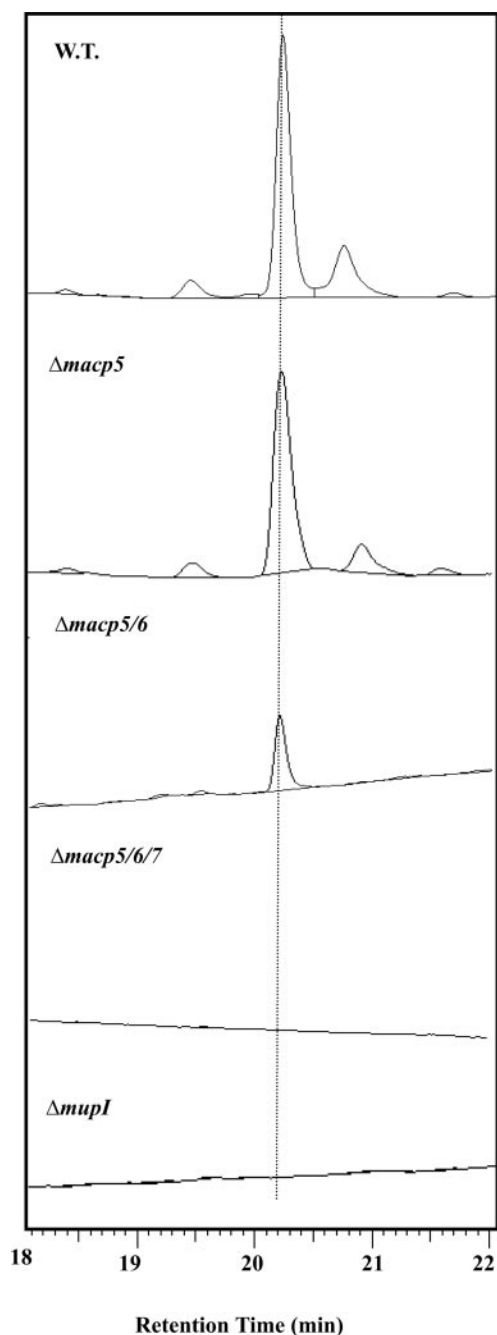


FIG. 4. The HPLC analysis showing the elution profiles when ACPs are successively deleted from the triplet cluster.

the $\Delta macp5/6/7$ once again was similar to the negative *mupI* mutant (Fig. 3, B and C). Pairwise deletions constructed within the mACP triplet cluster ($\Delta macp5/6$, $\Delta macp6/7$, $\Delta macp5/7$) did not abolish mupirocin production, but there was a significant decrease in the area of the zone of inhibition (Fig. 3C). The conclusion from this is that any one of the doublet and the triplet ACPs is sufficient for the formation of the polyketide chain but that reduction of the triplet cluster to a single mACP does have a quantitative effect on production.

Quantitative Analysis of Compounds Produced by Wild Type and Mutants—The compounds secreted into the supernatant of the wild type and mutant cultures were analyzed by reverse phase HPLC with an acetonitrile-water gradient elution on a silica column (Fig. 4). The system was calibrated with known amounts of mupirocin standard as described under "Experimental Procedures." *P. fluorescens* produces a mixture of PAs

(Fig. 1). Mupirocin, which is PA-A, elutes between 20 and 21 min and is seen as a single discrete peak on the chromatogram. The peak at ~19.5 min is predicted to be PA-B, and there are additional minor peaks seen after PA-A, which might be PA-C and PA-D, the other minor constituents of the antibiotic pool. All the single and pairwise deletions that retained antibacterial activity showed the same peaks as the wild type, whereas the double and the triple mutants ($\Delta macp3/4$, $\Delta macp5/6/7$), no longer produced any of the same compounds (as shown in Fig. 4). The time course of mupirocin production by the wild type strain was established by analyzing the samples collected between 16 and 64 h. Mupirocin production reached a plateau after 18 h, and the level then remained more or less constant until 64 h. The variables were reduced to a minimum while performing the quantitative analysis of mutants by equalizing the optical density of the seed cultures and maintaining similar culture conditions. The average area under the PA-A peaks of two clones of wild type and each mutant was taken at each time point. Production from the mutant cultures between 18–64 h was also analyzed and found to follow the same kinetics as seen for the wild type, reaching a plateau at ~18 h. The area under the PA-A peak was used as an estimate of the amount of antibiotic produced. As the peak areas were more or less constant once the plateau was reached, all of the peak areas were pooled to maximize the statistical significance of the results.

With the doublet mACPs in MmpA the deletion of either mACP3 and mACP4 had a similar effect. The $\Delta macp3$ and $\Delta macp4$ mutant strains produced 0.68 ± 0.03 and 0.62 ± 0.07 of the PA-A level of the wild type, the reduction being highly significant ($p < 0.001$) (Fig. 5A). With the triplet mACP cluster in MmpB the $\Delta macp5$, $\Delta macp6$, and $\Delta macp7$ mutant strains showed 0.36 ± 0.05 , 0.32 ± 0.03 , and 0.25 ± 0.02 , respectively, when compared with wild type, which is a highly significant decrease in mupirocin production. The pairwise mutants $\Delta macp5/6$, $\Delta macp6/7$, and $\Delta macp5/7$ produced 0.11 ± 0.01 , 0.16 ± 0.01 , and 0.10 ± 0.02 , respectively, compared with wild type as shown in Fig. 5B, a further significant decrease. This observation is more or less consistent with the expected additive effect of a double deletion except for $\Delta macp6/7$ that showed higher production than for the other double knock-outs. However, it would not be surprising if there were variations in the activities of the different mACPs especially because as argued below mACP5 may work in series rather than in parallel with mACP6 and mACP7.

The effect of removing mACP domains could be due not just to loss of 1 unit of ACP activity but could also be because of secondary effects on the remaining enzymic activities in MmpA or MmpB because of conformational changes in the protein. We therefore determined the effect of inactivating an ACP by a point mutation that would minimize structural changes. The point mutants *macp3S>A* (Mmp I S2664A), *macp4S>A* (MmpA S2769A), *macp5S>A* (Mmp II S1390A), *macp6S>A* (Mmp II S1478A) and *macp7S>A* (Mmp II S1574A) produced 0.61 ± 0.01 , 0.72 ± 0.03 , 0.03 ± 0.01 , 0.66 ± 0.03 , and 0.85 ± 0.13 when compared with the wild type as shown in Fig. 5. For mACP3 and mACP4 these results are generally in line with the deletion of whole mACP domains. The results were unexpected in the triplet cluster. Surprisingly *macp5S>A* showed no detectable mupirocin production, whereas the effect of the point mutations in *macp6* and *macp7* was much less than expected.

DISCUSSION

This study has focused on why there are tandemly repeated mACP domains in the Type I Mmps of the mupirocin biosynthetic cluster when normally only one such domain would be expected. Our strategy was to inactivate the mACP domains and determine the effects by antibacterial bioassay and by

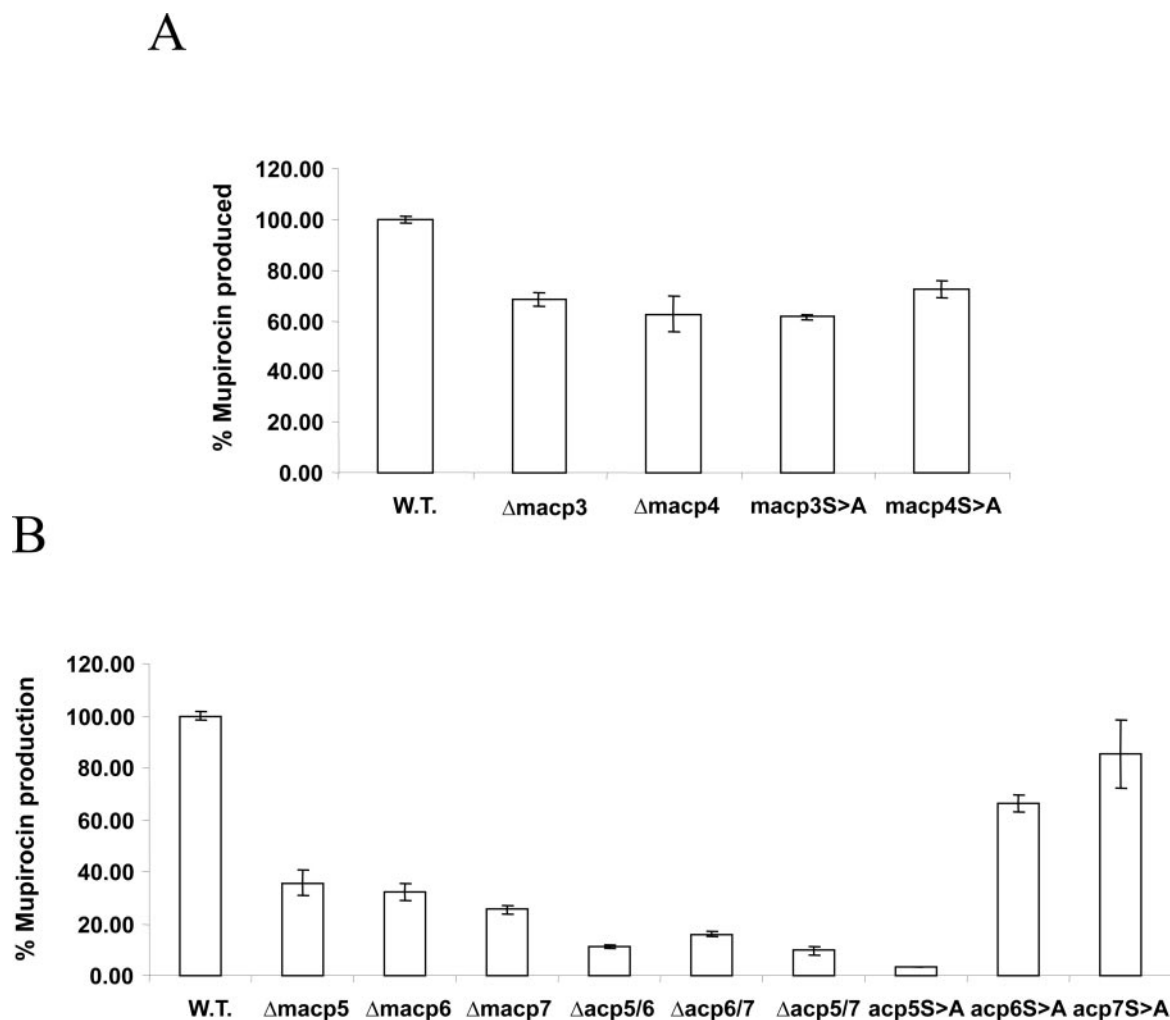


FIG. 5. Quantitative HPLC analysis of PA-A production in each of the mutants described in this paper.

HPLC. Although we were able to detect changes in clearing zone for the double deletions in the triplet cluster, the major conclusion from the plate bioassay was that each ACP in a cluster is functional and is capable of substituting for the other ACPs in that cluster. This seems much higher than the basal level of interchangeability expected between very different ACPs observed in previous work. For example, only weak complementation by the fatty acid synthase ACP was obtained for an ACP mutant in the *act* system of *Streptomyces coelicolor* where the level of identity was 31% identity between the ACPs (13). The finding that any of the ACP domains in the *mup* tandem clusters can suffice means that they can substitute for each other. It also indicates that these tandem clusters represent a single step in the biosynthetic pathway or that a single ACP can support more than one step.

HPLC analysis gave more reproducible results than the plate assay and provided the basis for the detailed quantitative analysis that we have performed. The consistent result was that the wild type with intact doublet or triplet clusters gave higher yields of mupirocin than any of the mutants and in the case of the triplet cluster progressive deletions of pairs generally gave bigger effects than deletion of single mACPs. Before detailed consideration of the quantitative data it is important to consider that the effect of deleting a domain could either be because of the loss of the function of that domain or could arise from conformational changes on the rest of the protein arising from the deletion. To address this issue a point mutation was introduced into the active sites of ACP3, ACP4, ACP5, ACP6,

and ACP7, resulting in the inability of the mutated ACPs to be converted into a holoenzyme by the addition of the phosphopantetheine arm. In the case of doublet ACPs this had a similar effect to that of deleting the whole domain. The similar loss of activity caused by deletion or point mutation for both mACP3 and mACP4 suggests that the deleterious effect on biosynthesis is because of loss of function of the ACP mutated and not because of a secondary effect. In the triplet cluster the fact that the effect of the deletions were approximately correlated to the number of ACP domains removed also suggests that the effect is because of a loss of ACP activity and not because of effects on neighboring domains. However, the differences between the production rates of the deletion *versus* substitution mutants of ACP6 and ACP7 might suggest that the deletions do affect the remaining activity of the protein. Nevertheless it is interesting that we find that the system is robust to deletions of adjacent domains. Previously it has been suggested that interactions with partner domains are very sensitive to sequence changes and it was proposed by Brun *et al.* (32) that modification of a function may be dependent on critical values of shared interactors (20%) and sequence identity (45%).

A critical deduction from the results obtained is that these tandem clusters represent points in the biosynthetic pathway where the presence of a single ACP would cause it to be a rate-limiting step. It may be no coincidence that these points are at the ends of the predicted monic acid and 9-hydroxynonanoic acid pathways. In fatty acid synthase systems chain termination is the rate-limiting step in fatty acid biosyn-

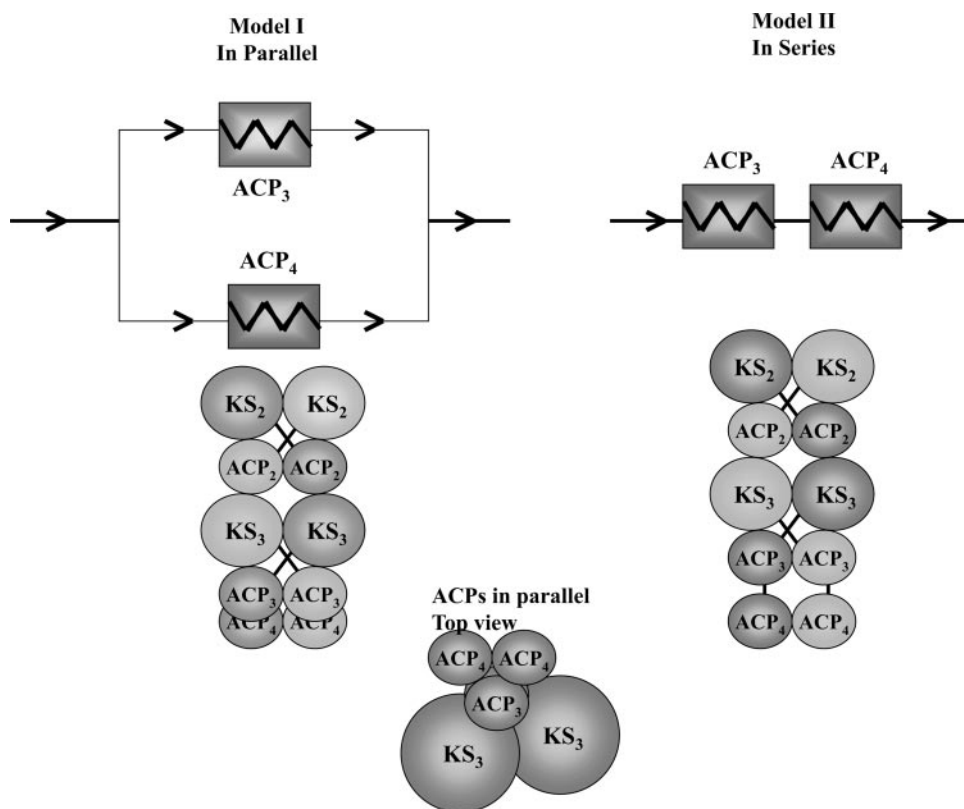


FIG. 6. Hypothetical dimeric MmpA models showing the arrangement of the doublet mACPs in series and in parallel. In *Model I* ACP₄ is arranged at an angle so that both the ACPs are within reach of the KS. *Model II* shows an in series arrangement where the domains are arranged like beads on a string.

thesis (33). Therefore the mupirocin biosynthesis machine may employ multiple ACPs to enhance the rate and overcome the rate-limiting step in the most economical way. The observation that interference at two different points can reduce biosynthesis confirms that normally the pathway works in balance with no single step being a severe bottleneck.

With the knowledge that each ACP domain is functional it is worth considering some theoretical models of how these tandem ACP clusters might function to increase mupirocin biosynthesis. In PKS systems ACPs have three general roles. The first role is to be charged by the extender unit, normally achieved by acyl transferases, although there is evidence for self-charging (34). The second role is to act as the acceptor for the growing chain when it is added to the extender. The third role is to carry the new chain to the modifying enzymes (ketoreductase, dehydratase, and enoyl reductase) before passing it onto the next KS or releasing it via the action of TE. If the rate-limiting step is charging of or release from the ACP, or length of time on the ACP, then having two ACPs that can function "in parallel" (to use an analogy from electricity circuits) may double the throughput (Fig. 6, *Model I*). Effectively in this model both ACPs would be identical and share the overall biosynthetic function. Alternatively, if it is specifically the processing of the chain while on the ACP that is rate-limiting rather than the movement on/off the ACP, then being able to shunt the chain onto another ACP might help. Thus one ACP might be charged and serve as the extender unit, whereas a second might receive the extended unit and act as the location for further modification. Such a model (Fig. 6, *Model II*) might be described as in series by contrast to the in parallel model. Such an in series model could arise from functional differences between the mACPs that are either inherent or arise from their position in the multiprotein complex. In Model I both ACPs would be equally accessible to KS and other domains, whereas in Model

II only the first ACP domain would need to be.

We will first focus on the data for the doublet cluster. MmpA is predicted to complete the biosynthesis of the monic acid backbone. In all the models both mACP₃ and mACP₄ are phosphopantetheinylated. In Model I (Fig. 6), both mACP₃ and mACP₄ would carry extender units, which condense with the growing chain in successive steps, so that ultimately there are two copies of the growing polyketide chain/unmodified monic acid on the two ACPs, thereby increasing the speed of the reaction. This model is consistent with the results obtained, because we see a comparable reduction when either ACP₃ or ACP₄ is deleted, although the level of biosynthesis is not quite reduced to 50% that of wild type. However, when a rate-limiting step is duplicated one does not necessarily get a doubling of overall rate, because another step may become rate-limiting. Conversely halving the number may not cause a 50% fall. The point mutations inactivating the ACPs further support this model, because they indicate that the presence of an inactive domain does not prevent access of KS to either mACP. As shown in Fig. 6 in the dimeric form of MmpA, for parallel functionality ACP₃ and ACP₄ have to be arranged one behind the other. This arrangement is consistent with the double helical Cambridge model proposed by Staunton and Weissman (8) where the ACP domain of the other subunit of the dimer interacts with the KS.

In Model II (Fig. 6), where an in series arrangement is visualized, only one unit of extender malonate is loaded onto one ACP of the doublet, possibly mACP₃. Decarboxylative condensation takes place between the growing polyketide chain and the extender and the condensed product is then passed onto mACP₄ where it can be acted upon by the putative hydroxymethylglutaryl-CoA-synthetase from the tailoring region to add on the C-15 group. The ACP₃, which is now free, can be loaded with another extender to condense with the incoming

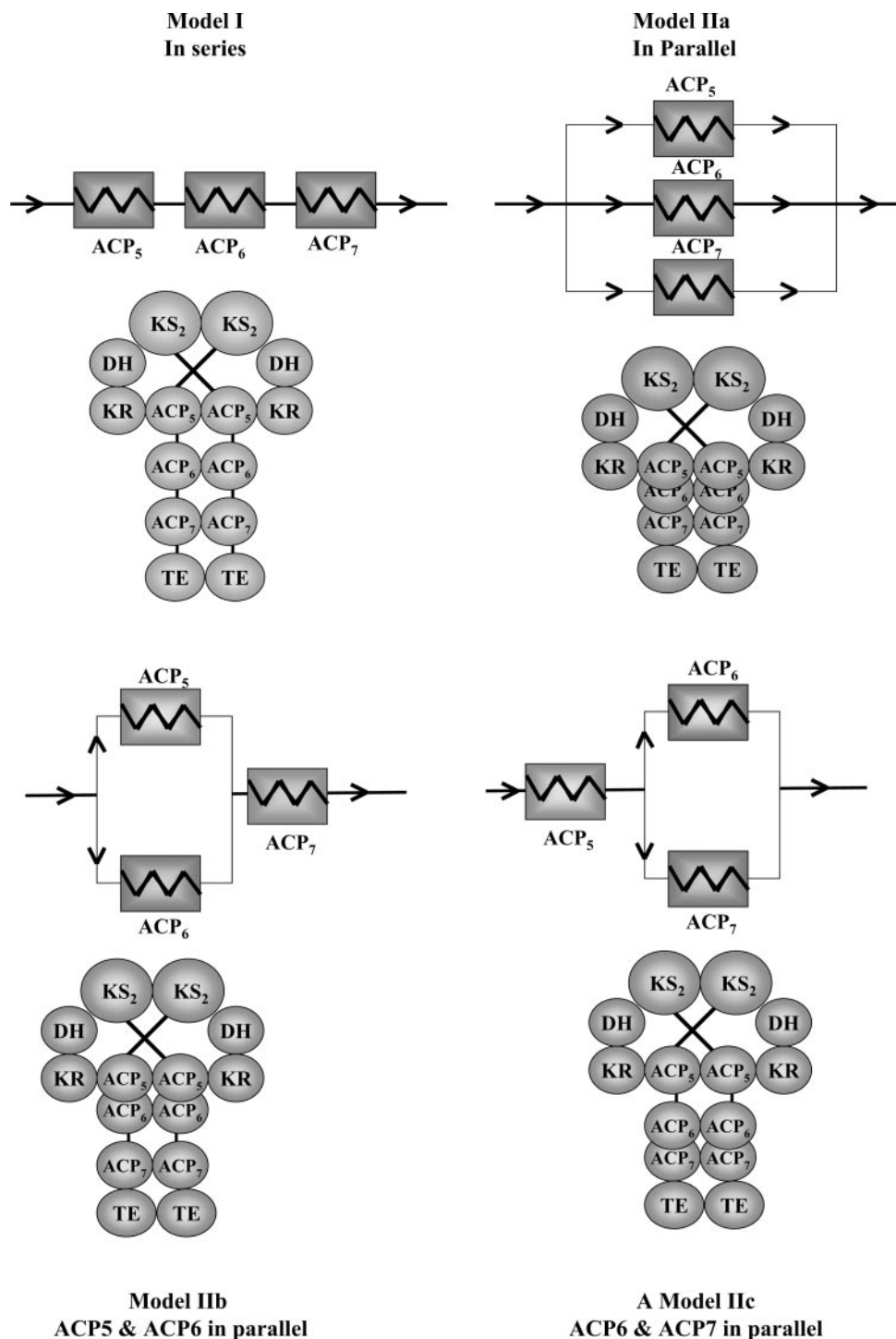


FIG. 7. Hypothetical dimeric MmpB models showing the arrangement of the triplet mACPs in series and in parallel. Models IIa, IIb, and IIc show different ways in which two of the three ACPs can be arranged in parallel. For Models IIb and IIc the single mACP could act first or second in the series.

polyketide chain, and the cycle continues. As the doublet ACPs are located at the end of the MmpA they might enjoy more conformational freedom to be acted upon, when the putative hydroxymethylglutaryl-CoA-synthetase associates with the multifunctional polypeptide to add the C-15 carbon. One more observation made from the analysis of ACPs from the mupirocin cluster is that the accessibility of the ACPs to its interacting partners also plays a crucial role in the synthesis of biological compounds. Thus, although flexible, the 20-Å maximal length of the phosphopantetheine arm restricts the transfer of the substrates to non-interacting domains. The results do not sup-

port such an in series arrangement.

Although we have only tentatively assigned MmpB a role in 9-hydroxynonanoic acid biosynthesis we can formulate a number of models for how the triplet cluster might work (Fig. 7). Following on from the discussion above we can see that the effect of point mutations suggest that the three mACPs do not all work in parallel (Fig. 7, Model IIa). Rather the results are consistent with Model IIc in which mACP6 and mACP7 work as a parallel pair in series with mACP5. Although Model IIc shows mACP5 working first, it could equally be that mACP6 and mACP7 are the first to be used. Models I and IIb are

inconsistent with the data, because in the former case inactivation of both mACP5 and mACP6 should have caused a severe block in the pathway, whereas in the latter model, inactivation of both mACP5 and mACP6 should have had only a small effect. Thus we propose that normally the reactions catalyzed by MmpB involve condensations followed by further modifications before ultimate release by TE. In favor of mACP6 and mACP7 performing the second step is the idea that formation of the acyl-enzyme intermediate on the TE is the rate-limiting step in both fatty acid synthase and PKS synthesis (35, 36). It would be plausible that to overcome the rate-limiting step either or both mACP6 and/or mACP7 may hold the intermediate while the mACP5 and KS catalyzes another condensation reaction. The need for two mACPs to have access to TE may not be a problem, because the flexibility of TE to dock or loop back to modules/ACPs away from the terminal module/ACP has been reported in picromycin/methymycin PKS (37). The transfer of intermediates from one ACP to another ACP is conceivable if the prosthetic groups are in proximity as ACP to ACP skipping has been previously reported in pikromycin (38) and an hybrid PKS of erythromycin and rapamycin (39).

Because this is the first time that a phenotype has been demonstrated for tandem ACPs, it is worth reviewing the systems where similar arrangements have been seen. The characteristic tandem ACPs of Claisen-type fungal naphthopyrone PKS of *A. nidulans* were studied by site-directed mutagenesis of both the active site serine residues to Cys or Ala. One single intact serine residue is sufficient for PKS function but whether both the serine residues are phosphopantetheinylated is yet to be determined. The quantitative effect such a mutation would have on naphthopyrone synthesis was not established (14). Tandemly duplicated ACPs are found in a few PKS, but their significance is not clear. For the twin ACPs of sterigmatocystin iterative Type I PKS in *A. nidulans*, it was speculated that one attaches the starter unit to KS and the other holds the malonate extender unit. Many other fungal PKSs, however, have a single ACP (15). It was reported in the R1128 anthraquinone-like estrogen receptor antagonist gene cluster that the presence of two acyl carrier proteins is because of the two sets of ketosynthases and acyl transferases with which the ACPs might interact in loading the unusual primer unit (40). The ACPs of R1128 gene cluster are, however, not tandemly repeated and are divergently transcribed unlike *mup* ACPs.

That the level of ACP activity can be rate-limiting has been established in a number of systems. Quantitative differences in hybrid PKS products were attributed to the differences in the upstream ribosomal binding sites, which probably affect the level of expression or efficiency of translation. The strong quantitative variation in total aromatic polyketide production was found to be either because of limiting ACP concentration or some level of translational coupling (41). Many *in vitro* and *in vivo* studies have confirmed that by increasing ACP concentration the level of antibiotic production can be increased. Deletion of the Type II ACP gene in *Streptomyces* lead to the total loss of antibiotic production (42), whereas the introduction of multiple ACPs increased antibiotic production (43). Introduction of the tetracenomycin ACP gene on high copy number plasmid into wild type or *tcm*-blocked mutants of *Streptomyces glaucescens* lead to a 30-fold increase in TcmD3 production and up to 25–30% increase in TcmC production (44). Crosby *et al.* (45) reported that when the amount of purified *act* ACP was doubled in an assay of malonyl transfer, the incorporation of [2-¹⁴C]malonate, which acts as the extender unit, was also doubled. Increasing the malonate concentration, however, did not result in malonate incorporation.

These examples are all from Type II systems in which the molar ratio of ACP to KS can be increased at the level of gene expression. In Type I systems, where the stoichiometry is fixed, the only way to increase the ACP activity is either by altering catalytic properties of an ACP domain or by increasing the number of domains with that activity as observed here. A single duplication event would presumably be more straightforward than multiple point mutations. The fact that the members of the *mup* tandem clusters represent groups of closest relatives, however, suggests that divergence then took place driven by the instability of directly repeated DNA sequences. It is interesting that the systems generated by these duplications seem to behave in such different ways, providing both in parallel and in series arrangements. This leads on to further questions about how location in the triplet cluster determines its function and what the constraints are that exist when trying to pack modules next to each other in a multifunctional polypeptide. As mentioned above the linkers between the mACP domains appear to be quite short (12 amino acids), but structural studies on other ACPs (46–49) indicate that the N and C termini are quite close together and flexible so even with this constraint it should be possible to pack and orient multiple domains to face the ketosynthase as well as other synthetic functions, at least when there are only two domains. The linkers between mACPs 5, 6, and 7 are even shorter (3 amino acids) than between mACP3 and mACP4. This reduced flexibility combined with there being three ACP domains may result in packing constraints that preclude a completely in parallel system so that it is inevitable that the MmpB mACPs behave as if they work partly in series when S→A amino acid substitution is used to inactivate modules. Although it seems clear therefore that the mechanistic adaptation provided by the triplet cluster is obviously not the same as that provided by the doublet, one of the challenges for the future will be to uncover why this arrangement increases the capacity of the biosynthetic pathway. Current studies are focused on this and other questions related to further increasing our understanding of the limitations on flux through the Mup pathway.

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