Common biosynthetic origins for polycyclic tetramate macrolactams from phylogenetically diverse bacteria

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A combination of small molecule chemistry, biosynthetic analysis, and genome mining has revealed the unexpected conservation of polycyclic tetramate macrolactam biosynthetic loci in diverse bacteria. Initially our chemical analysis of a *Streptomyces* strain associated with the southern pine beetle led to the discovery of frontalamides A and B, two previously undescribed members of this antibiotic family. Genome analyses and genetic manipulation of the producing organism led to the identification of the frontalamide biosynthetic gene cluster and several biosynthetic intermediates. The biosynthetic locus for the frontalamides' mixed polyketide/amino acid structure encodes a hybrid polyketide synthase nonribosomal peptide synthetase (PKS-NRPS), which resembles iterative enzymes known in fungi. No such mixed iterative PKS-NRPS enzymes have been characterized in bacteria. Genome-mining efforts revealed strikingly conserved frontalamide-like biosynthetic clusters in the genomes of phylogenetically diverse bacteria ranging from proteobacteria to actinomycetes. Screens for environmental actinomycete isolates carrying frontalamide-like biosynthetic loci led to the isolation of a number of positive strains, the majority of which produced candidate frontalamide-like compounds under suitable growth conditions. These results establish the prevalence of frontalamide-like gene clusters in diverse bacterial types, with medicinally important *Streptomyces* species being particularly enriched.

biosynthesis | frontalamide | genome-mining | Streptomyces | tetramic acid

Actinomycete bacteria produce the majority of antibiotics used in human and veterinary medicine. Though typically isolated from terrestrial and marine sediments, actinomycete symbionts of insects are becoming popular research targets because these insect-associated strains can provide potential sources of new antibiotics and insights into the chemical ecology of complex symbiotic relationships (1–3).

The southern pine beetle (SPB, *Dendroctonus frontalis*) hosts at least two such actinomycete strains. SPBs, which devastate large tracts of pine trees with consequent economic loss and local environmental change (4), engage in a multilateral symbiosis with two fungi the beetles introduce into their host trees. One fungus (*Entomocorticium* sp.) is carried in a specialized anatomical compartment within the beetle; the other (*Ophiostoma minus*) is associated with the beetle's exterior. *Entomocorticium* serves as the SPB larvae's food source and is essential for survival, while *O. minus*, which can outcompete the food fungus, threatens it (5).

Recent studies suggest SPBs use actinomycetes to promote the survival of their food fungi through the chemical control of *O. minus*. SPBs carry two distinct but closely related *Streptomyces* strains (6): *Streptomyces* sp. SPB74 (~60% prevalence) produces the antifungal mycangimycin (7), which preferentially inhibits the antagonistic fungus (6), and *Streptomyces* sp. SPB78 (~40%), which displayed no activity in Petri dish competition assays with the fungi. In spite of its frequent occurrence, SPB78 currently has no identifiable role in the symbiosis.

We now describe the results of our continued investigations on SPB78. Under certain culturing conditions, it produces two new antifungals as part of a complex of related molecules. Two of these, frontalamides A and B, have been purified and structurally characterized. The frontalamides were found to be similar to a number of known polycyclic tetramate macrolactams (Fig. 1), suggesting a common biosynthetic origin. Although no complete biosynthetic locus had ever been sequenced for any of these compounds, we were able to use a recently described fragment of the HSAF biosynthetic cluster (8) and molecular genetics to identify the biosynthetic locus of the frontalamides. This effort additionally led to three biosynthetic intermediates and their structures. Genome mining with frontalamide biosynthetic genes revealed a surprisingly large number of similar gene clusters in phylogenetically diverse bacteria, ranging from other actinomycetes to more distantly related γ-proteobacteria. Our analyses suggest that the polycyclic tetramic-acid macrolactam family originates from an unusual hybrid iterative PKS/NRPS pathway similar to that employed by eukaryal fungi but not previously recognized as such in bacteria. Further, our biosynthetic analysis led to probes with which to screen for frontalamide-like gene clusters, and these screens indicated that environmental *Streptomyces* strains harboring frontalamide-type biosynthetic genes are easily isolated from local soils. This genetic potential was assessed by culturing strains under suitable conditions, which revealed the production of apparent frontalamide analogs. Taken together, our results establish that a significant percentage of these medicinally important organisms likely share the ability to produce these compounds.

Results and Discussion

Discovery of frontalamides A and B. *Streptomyces* sp. SPB78 was grown on several different media and ethyl acetate extracts of the media were screened by LC/MS for the production of secondary metabolites. Comparing these extracts, we found that cultivation on YMS (9) agar plates led to the production of compounds not seen under other growth conditions. These compounds were produced as a complex of structurally related molecules, showing similar UV chromophores, retention times, and molecular masses. They exhibit UV maxima around 220 and 323 nm, which resembled the spectra of the known polycyclic tetramate macrolactams maltophilin (10) and ikarugamycin (11) (Fig. 1). Two members

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Data deposition: Sequencing results closing a gap between contigs NZ_ACEU01000453 and NZ_ACEU01000454 in the *Streptomyces* sp. SPB78 draft genome were deposited into GenBank under accession number GU722189. Sequences of the 16S rRNA genes from environmental isolates harboring *ftd* gene clusters, and their respective *ftd* screening amplicons, were deposited into GenBank under accession numbers GU722169-GU722188.

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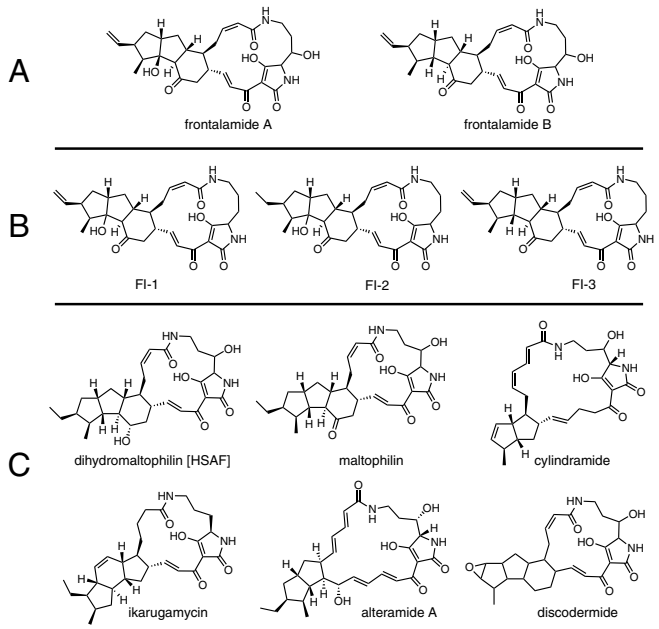

Fig. 1. Bacterial polycyclic tetramate macrolactams. (*A*) Structures of the newly isolated frontalamides. (*B*) Structures of the frontalamide biosynthetic intermediates FI-1, FI-2, FI-3. (*C*) Known bacterial polycyclic tetramate macrolactams with structures similar to the frontalamides.

of the complex were purified and structurally characterized as described in *Methods*, and the combined data (*SI Appendix*, Table S1 and Figs. S1 and S2) support the structures assigned to frontalamides A and B (Fig. 1). The frontalamides were named in recognition of *D. frontalis*, the insect from which *Streptomyces* sp. SPB78 was originally isolated.

The frontalamides are closely related to a number of previously described polycyclic tetramate macrolactam antibiotics including dihydromaltophilin (12), maltophilin (10), cylindramide (13), ikarugamycin (11), alteramide (14), and discodermide (15). Of these, the frontalamides are distinguished by an unusual terminal olefin. Little is known about the biosyntheses of any of these compounds, which is surprising because they are produced by a wide range of phylogenetically diverse bacteria including γ-proteobacteria [*Alteromonas* (14), *Stenotrophomonas* (10), *Lysobacter* (8)], actinomycetes [*Streptomyces* (11, 12)], and as-of-yet unidentified symbionts of marine sponges (13, 15).

Identification of the Frontalamide Biosynthetic Locus and Bioactivity of the Frontalamides. A fragment of the dihydromaltophilin (or HSAF) biosynthetic locus was recently identified and sequenced in the biocontrol strain *Lysobacter enzymogenes* (8). Because the frontalamides are structurally similar to HSAF, it seemed likely that they arose from similar biosynthetic origins despite the considerable evolutionary distance between the *Lysobacter* and *Streptomyces* genera. Four genes make up the incomplete HSAF locus, and interrogating the *Streptomyces* sp. SPB78 draft genome with two of these, which encode arginase and ferredoxin-like proteins, revealed no significant homologs. However, the other two genes from the *Lysobacter* locus, annotated as a sterol desaturase and hybrid PKS-NRPS synthetase, were found in the SPB78 genome with conserved synteny.

We established a genetic system in strain SPB78 to facilitate the identification of the frontalamide biosynthetic cluster. Gene deletions were produced using a markerless scheme to minimize potential polarity effects that can complicate downstream analyses. The deletion method required the isolation of an SPB78 *rpsL* point mutant (JV141), and before this strain was used to identify a frontalamide locus, we confirmed its ability to produce frontalamides (Fig. 2). Verification was necessary because *rpsL* mutations can significantly affect *Streptomyces* secondary metabolite production (16).

SPB78 *rpsL* strains deleted for homologs of the *Lysobacter* sterol desaturase (Δ*ftdA*, JV174) or hybrid PKS-NRPS (Δ*ftdB*, JV168) did not produce frontalamides A and B (Fig. 2), indicating that both are required for biosynthesis. The Δ*ftdA* mutant produces three major compounds related to the frontalamides but differing in relative abundance, retention times, and molecular masses. The Δ*ftdB* mutant produces no detectable frontalamide-like compounds.

The polycyclic tetramate macrolactams discodermide, dihydromaltophilin, and maltophilin are known antifungals, and wild-type SPB78 and its mutants were assayed for activity against *O. minus* in coculture (*SI Appendix*, Fig. S3). Our bioassays indicate that wild-type SPB78 and the *rpsL* mutant produce compounds that inhibit *O. minus*, whereas the *rpsL* Δ*ftdA*, and *rpsL* Δ*ftdB* double mutants did not produce clear zones of inhibition when plated against this organism. The effect of each SPB78 derivative on *O. minus* filament morphology was further examined under

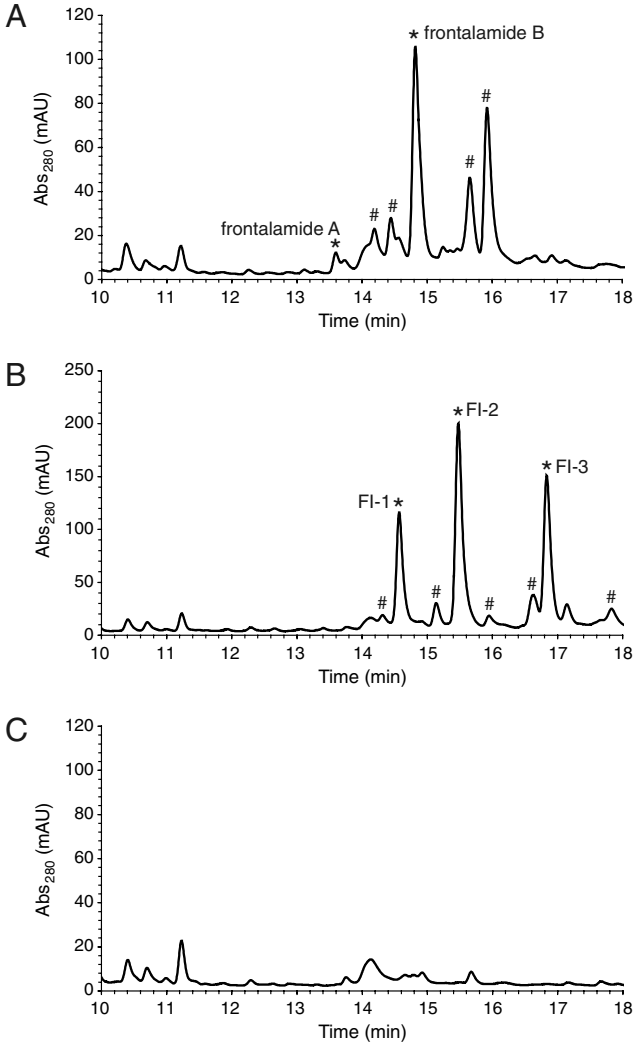

Fig. 2. LC/MS assays for frontalamide production. UV chromatographs were recorded at 280 nm. (*A*) LC/MS detection of frontalamides produced by a *Streptomyces* sp. SPB78 *rpsL* mutant. #s denote compounds with masses and UV spectra similar to frontalamides *A* and *B*. (*B*) Detection of frontalamide biosynthetic intermediates FI-1, FI-2 and FI-3 in a SPB78 *rpsL ftdA* double mutant. (*C*) Spectrum indicating the loss of frontalamide production in a SPB78 *rpsL ftdB* double mutant.

magnification. For *O. minus* growing in the presence of both the wild-type strain and the *rpsL* mutant, the fungal hyphae are deformed with irregular diameters and shapes. In the presence of the two SPB78 double mutants unable to produce frontalamides, the hyphae are indistinguishable from those grown in monoculture. The data indicate that frontalamides have antifungal activity against *O. minus* and that the biosynthetic intermediates secreted by the Δ*ftdA* mutant apparently lack the activity of the wild-type compounds.

Frontalamide-Type Gene Clusters Are Conserved Among Phylogenetically Diverse Bacterial Strains. The identification of polycyclic tetramate macrolactam biosynthetic genes, aside from the *ftdAB* homologs, was hampered in *Lysobacter* HSAF studies due to the lack of additional sequence data (8). The SPB78 draft genome, which is reasonably complete in the region containing the *ftdA* and *ftdB* genes, provided an opportunity to identify an intact biosynthetic locus for frontalamide-like compounds. In SPB78, the *ftdA* and *ftdB* genes are clustered with four others that are likely involved frontalamide biosynthesis due to an apparent operon-like arrangement (Fig. 3). The *ftdAB* genes comprise the most upstream flank of the cluster, and the remaining genes are named *ftdC-F*. Homology searches on these ORFs to predict possible biosynthetic roles led to the unexpected discovery that many recently sequenced bacterial genomes contain gene clusters with striking similarity to the frontalamide *ftdA-ftdF* cluster (Fig. 3). Many of the genomes containing these clusters are from *Streptomyces* (10 out of 13), but others include the marine actinomycete *Salinispora arenicola* (17) and the γ-proteobacterium *Saccharophagus degradans* (18). Of the *Streptomyces* genomes searched (including both draft and complete sequences), almost half (10 out of 21) contained *ftd* clusters.

The genes flanking the *ftd*-like loci are not conserved between species, but those located within the clusters display high homology and synteny. The *ftd*-like operons begin with *ftdA* homologs, which were variously annotated, depending on the source genome, as encoding sterol desaturases, fatty acid hydroxylases, or TRAP family dicarboxylate transporters despite high amino acid identity values (99-35% between all pairwise iterations). All of the FtdA homologs, save that from *S. arenicola*, contain putative amino-terminus transmembrane domains and three highly conserved histidine rich motifs (HX_3H, HX_2HH, and HX_2HH)—hallmarks of the fatty acid desaturase/alkane hydroxylase/xylene monooxygenase family of enzymes (19). Our assays showed that the SPB78 Δ*ftdA* mutant secretes biosynthetic intermediates, a result similar to that noted after insertional mutation of the *Lysobacter ftdA* homolog (8). The intermediates accumulating in the latter mutant were not characterized, so to assign a biosynthetic function to the SPB78 *ftdA* gene, the accumulated compounds from SPB78 Δ*ftdA* cultures were purified and structurally characterized. The combined chemical data for the intermediates (*SI Appendix*, Figs. S4, S5, and S6 and Table S2), designated FI-1, FI-2, and FI-3, led to the structures in Fig. 1. The FI intermediates are variable at more than one structural position, though the only conserved difference found between the three compounds is the lack of a hydroxyl group proximal to the tetramic-acid moiety of the compounds. This missing hydroxyl, together with the bioinformatics analysis above, is consistent with the role of FtdA as a hydroxylase. The FtdA-installed hydroxyl group is conserved in most known frontalamide-like compounds, and we therefore expect *ftdA* homologs encoded in other sequenced *ftd* loci to catalyze identical hydroxylations during the biosyntheses of their respective compounds.

Genes encoding hybrid PKS/NRPS synthetases (*ftdB* homologs) are found directly downstream of the *ftdA* homologs in the clusters. Comparisons of FtdB homologs' amino acid sequences indicate significant amino acid identity (53–91% over proteins ranging from 3,101–3,189 total residues). Each protein also contains the

Fig. 3. Open reading frame (ORF) map of the frontalamide biosynthetic (*ftd*) locus from *Streptomyces* sp. SPB78 compared to *ftd*-like clusters sourced from sequenced bacterial genomes. Each ORF is color-coded to designate *ftd* orthologs. Those shown in gray encode for alkane hydroxylases, red for hybrid PKS-NRPS synthetases, green for phytoene dehydrogenases, blue for zinc-dependent alcohol dehydrogenases, and yellow for cytochrome P450s. ORFs shown in black do not have homologs conserved between different species. The *S. clavuligerus* and *S.* sp. Mg1 clusters are shown as fragments because their draft genome sequences are incomplete in this region. The bracketed ORFS at the right flank of the *L. enzymogenes* cluster indicate those not analyzed during the initial characterization of this locus (8). These genes were released directly to GenBank without accompanying analysis.

hybrid PKS-NRPS catalytic domain order (KS-AT-DH-KR-ACP-C-A-T-TE) noted during studies on the partial dihydromaltophilin cluster (8). Based on their structures, a number of researchers working on frontalamide-like polycyclic tetramate macrolactams postulated the biosynthetic incorporation of ornithine or β-hydroxyornithine (12, 14, 15). This idea was further supported after ornithine-selective residues were found in the amino acid adenylation domain of the dihydromaltophilin FtdB

homolog (8). We analyzed these residues in the adenylation domain of each FtdB homolog using established methods (20, 21) and found that all contain conserved motifs characteristic of ornithine binding pockets (DVGE[V/I])GS[I/V]DK, *SI Appendix*, Table S3).

In the SPB78 *ftd* locus, two genes (*ftdC* and *ftdD*) similar to those encoding phytoene desaturase family enzymes, are found directly downstream of *ftdB*. Both genes are also found in most of the other *ftd*-like loci, except for the *S. degradans* and *S. arenicola* clusters, which contain only one *ftdCD*-type gene. The inferred translation products of these genes were compared in all pairwise combinations. To help deduce possible biosynthetic roles, sequences of the related enzymes phytoene desaturase (CrtI), diapophytoene desaturase (CrtN), and carotenoid isomerase (F4H5.10) were also included in the comparisons. The results (*SI Appendix*, Fig. S7) indicate that FtdC homologs, which have 62–99% identity between FtdC homologs sourced from different organisms, are more similar to FtdCs from other organisms than they are to FtdD homologs from the same organism. The same is also true of the FtdD homologs (having 42–100% identity between different organisms), with all being more similar to each other than to any FtdC homologs. The single *S. degradans* and *S. arenicola ftdCD*-type translation products are most similar to FtdD homologs. These results indicate that FtdC and FtdD, despite belonging to the same family of desaturases, are clearly distinct and likely catalyze different reactions. The proteins encoded by the *ftdCD* genes, which are similar to enzymes known to install, reduce, or isomerize double bonds, probably perform similar functions during the biosynthesis of frontalamide-like compounds.

The last two genes in the SPB78 frontalamide cluster are *ftdE* and *ftdF*. They are conserved in most of the *Streptomyces ftd*-like clusters, where they encode enzymes with sequence similarity to zinc-binding alcohol dehydrogenases and p450 hydroxylases, respectively. Like the rest of the proteins encoded in the conserved *ftd* loci, the homologs from each cluster share considerable amino acid identity (63–100% for FtdEs and 34–98% for FtdFs). Homologs of both *ftdE* and *ftdF* are notably absent from the *S. degradans* cluster, whereas the *S. arenicola*, *Streptomyces* sp. Mg1, and *S. clavuligerus* clusters are missing an *ftdE* or *ftdF* homolog. Given their similarity to enzymes that perform hydroxylation reactions or change carbonyl redox states, these enzymes likely perform oxidative tailoring reactions during frontalamide biosynthesis. For example, the alcohol and carbonyl groups located on the 5,5,6 ring system of frontalamide A are not found in positions characteristic of polyketide elongation chemistry, and it is plausible they were installed or modified using FtdE/FtdF–type enzymes.

The most surprising feature of the *ftd* clusters is the lack of additional PKS-type genes associated with them. Their absence is particularly intriguing because if the FtdB-type enzymes were to function modularly as postulated by Yu et al. (8), then the enzyme's polyketide synthase domains KS-AT-DH-KR-ACP would be responsible for the activation of a malonyl group, its subsequent decarboxylation, and incorporation into a growing polyketide backbone. (For a review on PKS and NRPS enzymology, see ref. 22). Likewise, the C-A-T-TE NRPS domains of the FtdB enzymes should activate an ornithine residue and ligate it to an ACP domain-tethered polyketide before hydrolysis of the polyketide-peptide product from the enzyme by the TE domain. The net function of the FtdB enzyme would then be to elaborate a relatively small portion of the backbone of frontalamide-like compounds. This analysis led previous authors to predict that the complete HSAF cluster would contain the additional PKS enzymes needed to synthesize the remainder of the polyketide chains (8). We expected the same to be true for the frontalamides. These expectations were further bolstered by the observation that bacterial tetramic-acid polyketides such as α-lipomycin are produced by modular PKS and NRPS enzymology, requiring multiple PKS enzymes (23). However, genome searches in the vicinity of *ftd* clusters revealed no juxtaposed PKS genes and left unresolved the issue of how the remainder of the frontalamide/HSAF-type backbone is biosynthesized.

The lack of additional PKS enzymes to produce the remainder of the polyketide backbone might be rationalized if FtdB-type enzymes function in a fashion similar to certain fungal hybrid PKS-NRPS enzymes, such as those involved in tenellin and cyclopiazonic acid biosyntheses. These eukaryal hybrid enzymes function iteratively in the PKS portion of the enzyme and noniteratively in the NRPS subunits (24). The fungal enzymes display a catalytic domain arrangement with clear similarity to that of FtdB (Fig. 4). An outstanding difference between the two is the presence of a "reductase"-type (R) thioesterase domain in the fungal enzymes, whereas bacterial FtdB homologs contain type I thioesterase domains (TE). Fungal R domains were recently shown to catalyze Dieckmann cyclizations that form tetramate moieties during thioester release (25, 26). If FtdB-type enzymes function as suggested above, the TE domains of these enzymes likely catalyze the same type of reaction and form the tetramate found in frontalamide-type compounds. Although the NRPS involved in the bacterial biosynthesis of α-lipomycin also does not contain an R domain to direct the requisite cyclization event, the compound's biosynthetic locus contains a free-standing TE domain with experimentally undefined function. Further investigation is required to determine the mechanistic origins of bacterial tetramate moieties, and if TE domains are involved in their catalyses.

If the above predictions regarding FtdB catalysis are correct, then these enzymes would have a unique ability to catalyze a second round of iterative polyketide synthesis before condensation with the δ-amino group originating from ornithine (Fig. 4). A Dieckmann cyclization would render the backbone complete, with concomitant product release, though it remains unclear how the 5,5,6 polycycle of the frontalamides form. This biosynthetic feature will remain unsolved until FtdB's polyketide intermediates have been structurally defined or the enzyme's activity has been reconstituted in vitro. We anticipate that like the fungal PKS/NRPS involved in tenellin biosynthesis, which requires a enoyl reductase encoded *in trans* to modulate both the redox state of the polyketide product and its correct chain length, other *ftd* cluster enzymes might be required to correctly modify the FtdB polyketide product for correct cyclization/maturation.

Screens for Environmental Frontalamide-Like Compound Production. Having identified *ftd*-type clusters in a number of sequenced bacterial genomes, we investigated their occurrence in environmental isolates. To this end, a degenerate primer-based PCR screen was designed to specifically detect the highly conserved *ftdA-ftdB* gene arrangement of *ftd*-type clusters. The resulting primers were tested for efficacy on four *Streptomyces* strains known to have these clusters. The genome sequences of two of these strains (SPB74 and SPB78) contain complete *ftd* loci, but the other two (*Streptomyces* sp. Mg1 and *S. clavuligerus*) have unfinished sequences in the region of interest (Fig. 3). The resulting PCR products were sequenced, confirming the utility of our primers to detect the desired clusters.

A total of 76 environmental actinomycetes were isolated from local garden and forest soils and screened as described above, and 15 isolates gave strong positive results. The 16S rRNA genes were amplified from each to aid in strain identification and the *ftd* screening products were sequenced to gauge cluster diversity. Comparing the 16S data against the GenBank database showed that all positive isolates most closely resembled *Streptomyces* species (*SI Appendix*, Table S4). Although some of these isolates showed nearly identical 16S rDNA sequences, differences in the sequenced *ftdA-ftdB* amplicons indicated they were not clonal. Of the original 15 strains, 10 were chosen for further analyses

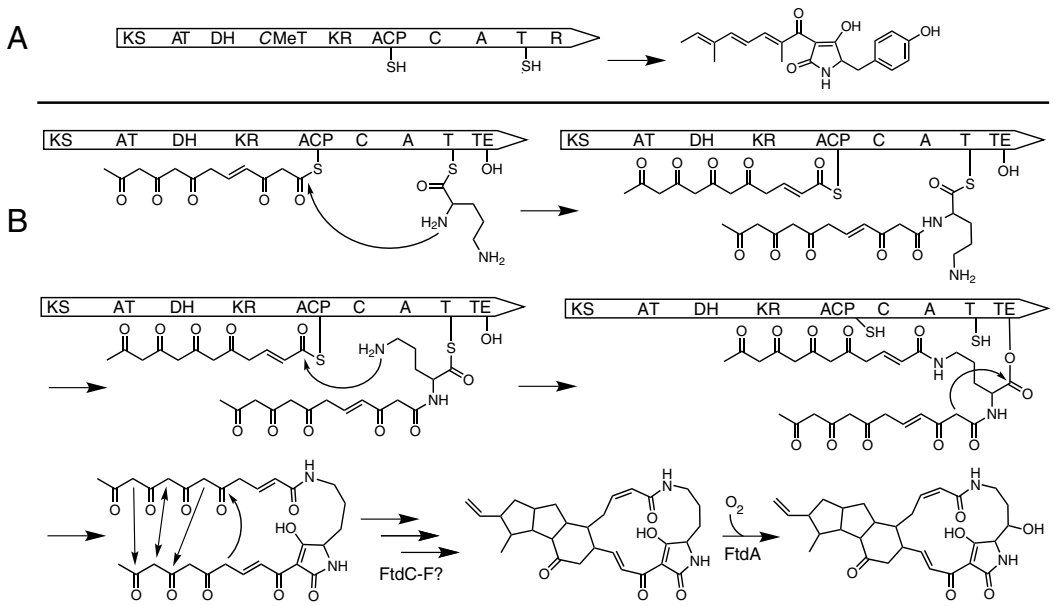

Fig. 4. Hypothetical model for frontalamide biosynthesis using a fungal-type hybrid PKS-NRPS mechanism. (*A*) Domain structure of the iterative type I PKS-NRPS hybrid enzyme TenS from the fungus *Beuveria bassiana*. Also shown is the mixed polyketide-amino acid tetramate product of TenS, prototenellin A. Note TenS iteratively condenses one acetyl-CoA and four malonyl-CoA substrates before incorporating a single tyrosine residue during the formation of protenellin A. (*B*) Homology-based biosynthetic model for the production of the mixed polyketide-amino acid tetramate backbone of the frontalamides by FtdB. Using an iterative mechanism to similar to that employed by the fungal enzymes, FtdB would catalyze two rounds of polyketide synthesis, and the two chains are subsequently condensed to the two free amines of ornithine. A Dieckmann cyclization could form the tetramic-acid moiety during release of the mixed polyketide-amino acid product from the FtdB synthetase. The roles of FtdC-FtdF await experimental elucidation but could plausibly be involved in redox tailoring of the FtdB product or for modification of the polyketide chains to direct the cyclizations that produce the 5, 5, 6 polycycle. This scheme represents a possible pathway for frontalamide biosyntheses; future studies are required. Our genetic data establish the hydroxylase role for FtdA, but we cannot ascertain the timing of this reaction during biosynthesis.

(JV176-JV182; JV184-JV86). Each of these strains was cultivated on four different growth media in parallel with *Streptomyces* sp. SPB74. The latter strain is the source of mycangimycin that, like SPB78, was originally isolated from *D. frontalis* beetles (7). SPB74 was included in the study because the experiments that yielded mycangimycin did not show any frontalamide production despite the later identification of an *ftd* cluster in its genome. The production of frontalamide-like compounds by the strains was evaluated using LC/MS. After comparing UV spectra, mass values, and retention times, the results suggest that eight of the tested strains, including SPB74, likely produce compounds with structures similar to the frontalamides (*SI Appendix*, Figs. S8 and S9). Additional experiments are underway to confirm if any of these compounds represent new polycyclic tetramate macrolactam structural members, but the results strongly suggest that once actinomycete bacteria harboring *ftd* type are identified, conditions can be found under which they appear to produce the expected products.

The identification of conserved *ftd*-type gene clusters in multiple bacteria, together with results demonstrating that additional cluster-containing strains can be isolated and used to produce candidate compounds, indicates a broad distribution, especially in the antibiotic producing *Streptomyces*. We anticipate these clusters will be repeatedly encountered as more bacterial genomes are sequenced. The commonality of these clusters raises questions about how groups of organisms can produce families of structurally conserved antibiotics without generating widespread resistance by their target organisms. We and others have observed that frontalamide-like antibiotics are produced as a complex of structurally related compounds. If the environmental roles of these compounds are indeed to act as antifungals, this structural promiscuity might represent a naturally occurring form of combination therapy. The ability of a single pathway to give rise to a suite of related molecules is a relatively common, if not always appreciated, feature of biosynthetic pathways (27).

Our analyses suggest that frontalamide-like gene clusters encode for an unappreciated bacterial biosynthetic strategy that is shared among phylogentically diverse bacteria. Nearly 50% of genome-sequenced *Streptomyces* strains carry an *ftd* cluster, a striking indicator that there is much to learn regarding even common unassigned (or orphan) gene clusters in these medicinally important organisms. Further exploration of these compounds and their ecological roles might provide important lessons in chemical ecology and templates for new therapeutic agents.

Materials and Methods

Bacterial Strains, Culture Conditions, and Molecular Methods. Strains, media, culture conditions, and molecular cloning techniques used in this study are described in the *SI Appendix*.

Genetic Manipulation of *Streptomyces* sp. SPB78. Gene deletion mutants were created in *Streptomyces* sp. SPB78 using an *rpsL* dominance based counter-selection strategy (28). Design and construction of deletion cassettes used to carry out the unmarked deletion of the frontalamide biosynthetic genes *ftdA* and *ftdB* were carried out as previously described (29). Detailed methods used to construct these mutants can be found in the *SI Appendix*.

Purification and Structural Elucidation of the Frontalamides and Related Biosynthetic Intermediates. Frontalamides A and B (and biosynthetic intermediates FI-1 ~ FI-3) were isolated from YMS agar plates after innoculation with *Streptomyces* sp. SPB78 (or respective mutant strains thereof), extraction with ethyl acetate, and purification using standard HPLC or LC/MS methods. The structures of these compounds were solved using UV, IR, MS, 1-D NMR (^{1}H, ^{13}C, NOE), and 2-D NMR (gCOSY, TOCSY, NOESY, gHSQC and gHMBC) experiments. Further detail regarding these experiments can be found in the *SI Appendix*.

Bioassay of SPB78 and Deletion Mutants Against *Ophiostoma minus*. To assay the activity of SPB78 produced frontalamides against the fungus *Ophiostoma minus*, wild-type strain SPB78 and SPB78 mutants JV141, JV174, and JV168 were grown in a shaken MYG (29) liquid starter culture for 2 d at 30 °C. Aliquots (0.2 ml) of these cultures were spread for confluence on YMS agar

and incubated for 5 d at 30 °C. Agar plugs (6 mm) were cut from these plates and applied to the periphery of a YMS agar plate. To the center of this plate was applied a YMS agar core inoculated with *O. minus*. The bioassay plate was incubated for 9 d to allow the *O. minus* fungus to spread over the plate's surface, and the gross bacterial-fungal interactions were recorded with a flatbed digital scanner. Photomicrographs to record *O. minus* cellular morphology in the presence of the test strains were recorded on a Zeiss Axioscope A1 (Zeiss A-Plan 5X objective) equipped with a Zeiss ICc1 camera. Scale bars were installed using Zeiss AxioVision Version 4.7 software.

Isolation and Screening of Environmental Actinomycetes for Frontalamide-Like Gene Clusters and Production of Related Polycyclic Tetramate Macrolactams. Two soil samples for the isolation of actinomycetes were obtained in New London County, CT. One sample was sourced from cultivated vegetable garden soil and the other from forest soil. The basic protocol used for the enrichment and isolation of actinomycetes from these samples was adapted from El-Nakeeb et al. (30) using AGS (30) or LTY (31) agar media with added cycloheximide (50 mg/L final concentration) to inhibit fungal growth.

Actinomycete isolates were screened by PCR using degenerate-code primers. The forward primers were designed to hybridize with conserved regions in the 3′ ends of *ftdA*-like genes and the reverse primers bind to conserved regions in the 5′ ends of the hybrid *ftdB*-like genes. The degenerate primer sets used to screen the isolates for *ftd*- like clusters were designed using BLOCKS (32) and CODEHOP (33) software. Isolates were screened via PCR after samples were prepared as recommended by Van Dessel et al. (34).

Environmental isolates thought to harbor putative frontalamide-like clusters after PCR analyses were spread to the following agar media: LTY (31), YMS (9), Hickey-Tresner agar (35), and ISP4 (Becton, Dickinson & Co., Sparks, MD). After allowing growth for 5 d (10 d for SPB74) at 30 °C, the growth media were extracted as described for the isolation of the frontalamides, and the resulting extracts were analyzed by LC/MS. Further detail can be found in the *SI Appendix*.

Accession Numbers of Source Files Used to Identify *ftd*-Like Gene Clusters in Bacterial Genomes. The following GenBank releases were sources of *ftd*-like gene clusters used in comparative analyses. Gene identification numbers delimiting the putative clusters are listed in parentheses if they were sourced from finished genome assemblies. *Lysobacter enzymogenes* HSAF partial cluster (8), GenBank accession EF028635.1; *Lysobacter enzymogenes* HSAF complete cluster, GenBank accession EF028635.2; *Streptomyces. albus* J1074 cluster GenBank accession ABYC01000481; *Salinispora arenicola* CNS-205 (Sare_2406-Sare_2409) (17) GenBank accession NC_009953; *Saccharophagus degradans* ATCC 43961 (Sde_3724-Sde_3726) (18) GenBank accession CP000282; *Streptomyces flavogriseus* ATCC 33331 GenBank accession NZ_ACZH01000010; *Streptomyces griseus* (Sgr_810-Sgr_815) (36) GenBank accession AP009493; *Streptomyces roseosporus* ATCC 11379 GenBank accession ABYX01000252; *Streptomyces clavuligerus* ATCC 27064 GenBank accession ABJH01000576; *Streptomyces* sp. Mg1 GenBank accession ABJF01000449; *Streptomyces* sp. SPB74 GenBank accession NZ_DS570564; *Streptomyces* sp. SPB78 GenBank accessions NZ_ACEU01000453 and NZ_ACEU01000454; *Streptomyces* sp. ActE GenBank accession ADFD01000006.1; *Streptomyces* sp. Act-1 GenBank accession ADFC01000012.1.

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