# Significant Natural Product Biosynthetic Potential of Actinorhizal Symbionts of the Genus *Frankia*, as Revealed by Comparative Genomic and Proteomic Analyses[∇]

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Bacteria of the genus *Frankia* are mycelium-forming actinomycetes that are found as nitrogen-fixing facultative symbionts of actinorhizal plants. Although soil-dwelling actinomycetes are well-known producers of bioactive compounds, the genus *Frankia* has largely gone uninvestigated for this potential. Bioinformatic analysis of the genome sequences of *Frankia* strains ACN14a, CcI3, and EAN1pec revealed an unexpected number of secondary metabolic biosynthesis gene clusters. Our analysis led to the identification of at least 65 biosynthetic gene clusters, the vast majority of which appear to be unique and for which products have not been observed or characterized. More than 25 secondary metabolite structures or structure fragments were predicted, and these are expected to include cyclic peptides, siderophores, pigments, signaling molecules, and specialized lipids. Outside the hopanoid gene locus, no cluster could be convincingly demonstrated to be responsible for the few secondary metabolites previously isolated from other *Frankia* strains. Few clusters were shared among the three species, demonstrating species-specific biosynthetic diversity. Proteomic analysis of *Frankia* sp. strains CcI3 and EAN1pec showed that significant and diverse secondary metabolic activity was expressed in laboratory cultures. In addition, several prominent signals in the mass range of peptide natural products were observed in *Frankia* sp. CcI3 by intact-cell matrix-assisted laser desorption-ionization mass spectrometry (MALDI-MS). This work supports the value of bioinformatic investigation in natural products biosynthesis using genomic information and presents a clear roadmap for natural products discovery in the *Frankia* genus.

The large bacterial phylum *Actinobacteria* harbors a diverse assemblage of high-G+C, Gram-positive bacteria that prosper in a wide range of environments (60). A notable feature of the microbes belonging to the order *Actinomycetales* is their metabolic versatility in the production of chemically diverse and biologically potent natural products (10). Genomic analyses have clarified the extent of their secondary metabolic proficiency, which is particularly wide ranging in those with genome sizes greater than 5 Mb (39). As exemplified by the genus *Streptomyces*, many soil-dwelling, filamentous actinomycetes are prolific natural product producers, secreting antimicrobial agents, signaling molecules, pigments, and other chemicals into their surroundings to thwart competition or condition their environment (17). While biosynthetic pathways to secondary metabolites are numerous and diverse, advances in the molecular rationale behind natural product assembly have allowed for the prediction of chemical classes and structures (15).

*Frankia* bacteria are mycelium-forming actinomycetes that are found as nitrogen-fixing symbionts in the root nodules of angiosperm plant species but can also survive as free-living soil bacteria (9, 14). These bacteria are developmentally complex, forming three cell types: vegetative hyphae, spores located in sporangia, and unique lipid-enveloped cellular structures called vesicles. Vesicles are formed inside the plant cells of the nodules or in culture under nitrogen-limiting conditions and act as specialized structures for nitrogen fixation process. These slow-growing microbes inhabit highly selective environments and are often closely associated with actinorhizal plant families with distinct host ranges. Genome sequence analysis

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of three strains with different host range specificities revealed large genomic variance in which genome size markedly expanded upon host plant diversification (40). Sizes varied from 5.43 Mb for the narrow-host-range *Frankia* strain CcI3 to 7.50 Mb for the medium-host-range *Frankia* strain ACN14a (ACN) to 8.98 Mb for the broad-host-range *Frankia* strain EAN1pec (EAN). Since the elucidation of these *Frankia* genomes, bioinformatic approaches have illuminated codon usage patterns (47), predicted secretosome profiles (35), and led to genome-guided studies on the *Frankia* transcriptome (3, 44) and proteome (1, 5, 33, 34). Genome mining also provides an opportunity to identify important physiology and metabolic functions, including secondary metabolism. This approach helped identify the auxin biosynthesis pathway used by *Frankia* strain CcI3 to drive nodule development in *Casuarina glauca* (41).

Beyond a few cases, *Frankia* natural product biochemistry has largely been unexplored and is ripe for genome-mining approaches. Quinonoid pigments of presumptive polyketide origin and a derivative of the calcium-binding streptomycete-produced calcimycin antibiotics, demethyl C-11 cezomycin, were isolated from *Frankia* strains ORS 020604 and AiPs1, respectively, and their structures determined as early examples of *Frankia*-based natural products (25, 30). The most well-studied area for *Frankia* has focused on the production of numerous oxidized and cyclopropanated fatty acids and triterpene hopanoids that differ in content between vegetative-growth cells and those in $N_2$-fixing vesicles (54). Hopanoids are a major component of the vesicle cell envelope and serve to protect nitrogenase from oxygen inactivation (11). Experiments with various *Frankia* cultures have furthermore indicated production of compounds with siderophore and antibiotic activities, though novel structures have not been reported (24). Discovery work using *Frankia* cultures is hampered by their slow growth and difficult laboratory manipulation (7). Hence, bioinformatics offers a glimpse into the *Frankia* secondary metabolome that may provide new insights into their relationships with higher plants. The goal of this study was to reveal the predicted *Frankia* secondary metabolome, including potential structures, and to show proof of concept for this approach. These predicted natural products would provide potential targets for future studies on plant-microbe interactions and other functions involved in *Frankia* physiology.

## MATERIALS AND METHODS

**Bioinformatic analysis.** Biosynthetic loci in *Frankia* strains ACN14a (GenBank accession no. CT573213.2), CcI3 (GenBank accession no. CP000249.1), and EAN1pec (GenBank accession no. CP000820.1) were identified using a BLAST-based method previously reported (55). Briefly, translated genes from each *Frankia* genome were compared to a collection of 20 "standard" secondary metabolism gene or domain sequences using the BLASTp algorithm. High local concentrations of hits (genes with a BLASTp error value less than E−07), as well as indicative single-target hits, were grouped with approximately 10 kb of nucleotide sequence upstream and downstream and labeled as putative biosynthetic gene clusters. Putative clusters were given species-specific designations (FA, *Frankia* strain ACN14a; FC, *Frankia* strain CcI3; and FE, *Frankia* strain EAN1pec). Further analysis of the clusters led to elimination of false secondary metabolism hits, leading to minor gaps in numbering. Open reading frames (ORFs) were verified using FramePlot (27). BLAST (4), CD-Search (32), and Pfam (8) were used for sequence analysis and prediction of gene functions, and Gene Neighborhood (36) and KEGG (29) were utilized for comparative analysis. Natural product-specific analysis methods (55) were used for determi-

nation of polyketide synthase (PKS) ketosynthase loading versus extension capability, acyltransferase substrate specificity, ketoreductase stereochemistry, nonribosomal peptide synthetase (NRPS) adenylation domain amino acid specificity, and condensation domain cyclization capability. As clear guidelines for the delineation of biosynthetic gene cluster boundaries do not exist, best estimates were made on the basis of functional analysis, G+C or codon bias, substantial (>~1-kb) gaps between ORFs, and/or the presence of transposition-associated genes and genes of unknown/unpredictable function.

**Culture conditions.** *Frankia* strains EAN1pec and CcI3 were grown and maintained in succinate and propionate growth medium, respectively, with $NH_4Cl$ as the nitrogen source, as described previously (52). Under $N_2$ conditions, cultures were grown in their respective growth media without $NH_4Cl$. For the $^{15}N$ labeling experiments, CcI3 cultures were grown 7 days in propionate growth medium containing 10 mM $^{15}NH_4Cl$ (100% $^{15}N$).

**Proteomic analysis.** Frozen pellets of EAN and CcI3 were resuspended in 100 mM Tris-HCl (pH 7.5) and sonicated. The crude lysate was centrifuged and the supernatant collected. Ammonium sulfate-precipitated proteins were resuspended in Tris-HCl, quantified by a Bradford assay (B6919; Sigma-Aldrich), and digested overnight (trypsin singles kit; Sigma-Aldrich). Mass spectrometry analysis was performed with an Agilent 1200 quaternary pump high-performance liquid chromatograph (HPLC) fitted with an in-house reversed-phase $C_{18}$ 100-μm by 5-μm fused silica capillary column and coupled with a Thermo Scientific LTQ XL mass spectrometer. The data were searched against the CcI3 (GenBank accession no. CP000249.1) and EAN (GenBank accession no. CP000820.1) genomes and a database of common contaminants (such as human keratin and porcine trypsin).

**Intact-cell (IC) MALDI-TOF analysis.** Regular and $^{15}N$ incubated cultures of *Frankia* sp. CcI3 were prepared as described above and frozen samples spun down in 1.5-ml Eppendorf tubes for 1 min at 10,000 rpm. Approximately 1 μl of each pellet was placed on a well of the matrix-assisted laser desorption ionization (MALDI) target plate (Bruker Microflex MSP 96 stainless steel target) and analyzed by MALDI-time of flight (TOF) mass spectrometry as previously described (20).

## RESULTS AND DISCUSSION

**Organization and general features of the *Frankia* secondary metabolome.** The sequenced *Frankia* strains maintain no plasmids, have similar numbers of protein-coding regions per kilobase (CcI3, 0.829; ACN, 0.905; and EAN, 0.882), and, despite the vast differences in chromosome size, appear very closely related on the basis of 16S rRNA sequence identity (each ~98 to 99% identical to the other) (40). In total, 65 secondary metabolism biosynthetic gene clusters from the *Frankia* genomic sequences were identified (Fig. 1). Of these, more than half were clusters for which chemical structure predictions of all or part of the products could be made by bioinformatic analysis alone. With the exception of the expected hopanoid-related biosynthetic genes (2, 18), no clusters could be unambiguously correlated with any of the few known secondary metabolites isolated from *Frankia* strains, and none of the *Frankia* modular PKS- or NRPS-containing gene clusters have previously been identified in other species. Despite the wide variability of chromosome sizes, clusters were evenly distributed between the strains, with roughly 20 found in each. Thus, at least in the case of the *Frankia* genomes available, genome size was not a predictor of biosynthetic potential (19). Brief descriptions and chromosomal locations of secondary metabolic gene clusters are cataloged in Table 1 and Fig. 1, respectively.

Presumed nonfunctional, fragmented biosynthetic clusters, such as those reported to occur at the unstable ends of *Streptomyces coelicolor* A3(2) and *S. avermitilis* linear chromosomes (39), were observed only in the smallest (for CcI3: FC01, FC03, FC13, and FC15b) and largest (for EAN: FE11, FE16, and FE23) chromosomes. Assumptions of nonfunctionality were
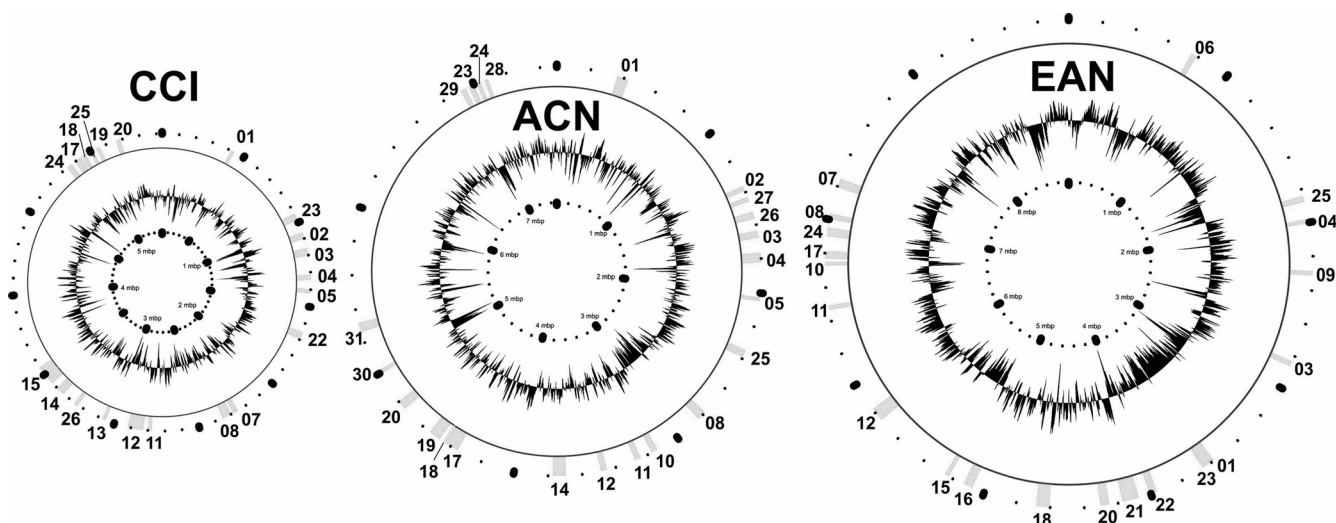
FIG. 1. Circular chromosomes of *Frankia* sp. HFPCcI3 (CCI), *Frankia* sp. ACN14a (ACN), and *Frankia* sp. EAN1pec (EAN), oriented to the *dnaA* gene. The inner ring indicates deviation of GC content from the genomic average. The outside outer ring shows the locations of secondary metabolic gene clusters that correlate with the cluster names provided in Table 1.

made on a variety of bases, most typically including lack of critical fragments of domains or active site residues. Although genome sequencing assembly issues should not be ruled out, it is possible that the presence of fragmented clusters are an artifact following the presumed expansion of the EAN chromosome or a possible contraction in the case of the CcI3 chromosome (40). Highlights of our findings are described below.

**Gene clusters associated with polyketide biosynthesis.** Biosynthetic gene clusters associated with polyketide-derived specialized lipids are a predominant theme in the putative *Frankia* secondary metabolome. Previous lipid studies revealed that *Frankia* $N_2$-fixing vesicles are replete with unknown long-chain polyhydroxy fatty acids as well as typical iso-branched and monounsaturated fatty acids (54). Many iterative PKS systems were observed in each genome, several containing genes with strong similarity to mycobacterial PKSs associated with the synthesis of mycocerosates (53) (FA02, FA11, and FE22; compound 1) (Fig. 2). Novel biosynthetic routes to similar molecules also exist, such as the type I modular PKSs in clusters FA01, FE18, and FE21, whose repetitive domain structures imply construction of more highly oxidized, differentially methylated analogs of mycocerosates (such as compounds 2, 3, and 4). These results indicate chemical similarities between the composition of the cell walls of the disease-causing mycobacteria (16, 23) and the plant symbiont *Frankia*. PKSs from clusters FA04, FC15a, and FE17 bear strong architectural similarity to polyunsaturated fatty acid (PUFA) biosynthetic systems from marine gammaproteobacteria (37), which may represent novel PUFA biochemistry in a terrestrial bacterium. While genes encoding acetyl-coenzyme A (CoA) carboxylase systems are also observed clustered in the *Frankia* PUFA PKSs, they are uniquely flanked by a number of genes suggestive of lipid metabolism. Thus, the *Frankia* clusters may code for the synthesis of modified PUFA products (compound 5).

The *Frankia* bacteria also contain antibiotic-like modular type I PKSs (e.g., FA17 and FE12), nonreducing iterative type

I PKSs (e.g., FE20; compound 6), heterodimeric type II PKSs associated with spore pigment (FA20, FC14, and FE03) and antibiotic (FC17) production, and homodimeric type III PKSs (FA24 and FE15) (Table 1). The FA17 and FE12 gene clusters encode large, modular type I PKS systems lacking typical terminal offloading thioesterase (TE) domains. Flanking the five PKS open reading frames of FA17 are genes homologous to *chlM* and *chlD1-4* from the *Streptomyces antibioticus* chlorothricin biosynthetic gene cluster (28). Taken together, these data suggest that FA17 codes for the biosynthesis of a novel spirotetronate antibiotic (50) such as compound 7 with programmed differences in the PKS-derived region. The FE12 locus rather harbors a smaller tetramodular PKS that appears to be primed with 3-amino-5-hydroxybenzoic acid (AHBA), characteristic of ansamycin polyketide antibiotics such as rifamycin (21). Genes encoding AHBA biosynthetic enzymes are not entirely contained in the FE12 locus but partially reside elsewhere in the genome in a conserved region (Franean1_1658 to -1664) common with CcI3 and ACN. FE12-associated polyketide extension with four methylmalonyl-CoA molecules followed by macrolactam and cyclic ether formation of the highly functionalized polyketide chain may yield the recently described *Saccharopolyspora cebuensis* macrolactams cebulactams A1 and A2 (compound 8), for which no biosynthetic studies have been reported (43).

**Gene clusters associated with nonribosomal peptide biosynthesis.** All three *Frankia* genomes maintain nonribosomal peptide synthetases (NRPSs) for siderophores and antibiotic-like cyclic peptides (31). Three *Frankia* NRPS-based siderophore biosynthetic pathways (FA19, FE01, and FE07) are expected to produce molecules containing iron-chelating residues such as phenols/catechols, oxazolines/thiazolines, and hydroxamates. In each case, the NRPS domain architecture suggests the sequential addition of salicylate or 2,3-dihydroxybenzoate followed by serine or cysteine and a further 3 to 6 amino acid residues to give compound 9 (FA19), compound 10 (FE01), and compound 11 (FE07). All three *Frankia* genomes harbor a

TABLE 1. Distribution, biosynthetic classification, and predicted products of the secondary metabolome of
*Frankia* strains ACN14a, CcI3, and EANpec1

| Strain and gene cluster | Approximate location | Type | Proposed product | Predicted structure[a] |
|---|---|---|---|---|
| *Frankia alni* ACN14a | | | | |
| FA01 | FRAAL0341–FRAAL0352 | Type I modular PKS | Specialized lipid | 2 |
| FA02 | FRAAL1275–FRAAL1282 | Type I PKS | Mycocerosate-like lipids | 1 |
| FA03 | FRAAL1549–FRAAL1558 | Type I iterative PKS | Unknown | Unk |
| FA04 | FRAAL1658–FRAAL1682 | Type I iterative PKS | PUFA | 5 |
| FA05 | FRAAL1880 | Quinone "NRPS" | Unknown quinones | 12 |
| FA08 | FRAAL2558–FRAAL2576 | Hybrid PKS-NRPS | Hybrid polyketide/peptide | 14 |
| FA10 | FRAAL2909–FRAAL2914 | Type I iterative PKS | Unknown, same as FA03 | Unk |
| FA11 | FRAAL2986–FRAAL2992 | Type I PKS | Mycocerosate-like lipids | 1 |
| FA12 | FRAAL3193–FRAAL3198 | Type I iterative PKS | PUFA | 5 |
| FA14 | FRAAL3421–FRAAL3473 | Type I PKS | Beta-hydroxy butyrate | Unk |
| FA17 | FRAAL4060–FRAAL4102 | Type I PKS | Chlorothricin-like ring system | 7 |
| FA18 | FRAAL4105 | Quinone "NRPS" | Unknown quinones | 12 |
| FA19 | FRAAL4152–FRAAL4172 | NRPS | Siderophore | 9 |
| FA20 | FRAAL4378–FRAAL4406 | Type II PKS | Spore pigment | Unk |
| FA23 | FRAAL6421–FRAAL6428 | Non-NRPS siderophore | Siderophore, same as FC24, FE06 | Unk |
| FA24 | FRAAL6457–FRAAL6460 | Type III PKS | Unknown, see also FE15 | Unk |
| FA25 | FRAAL2154–FRAAL2174 | Terpene synthase | Carotenoids | 20 |
| FA26 | FRAAL1427–FRAAL1449 | Terpene synthase | Hopanoids | 15-19 |
| FA27 | FRAAL1335–FRAAL1339 | Terpene cyclase | Pentalenene | 22 |
| FA28 | FRAAL6507 | Terpene cyclase | Geosmin | 21 |
| FA29 | FRAAL6371–FRAAL6381 | Phosphonate | Unknown | Unk |
| FA30 | FRAAL4919–FRAAL4922 | Ribosomal peptide | Microcin-like | Unk |
| FA31 | FRAAL4634–FRAAL4646 | Aminocyclitol | Cetoniacytone-like | Unk |
| | | | | |
| *Frankia* sp. CcI3 | | | | |
| FC01 | Francci3_365 | NRPS (PCP-TE didomain) | Fragmented/nonproducing cluster? | Unk |
| FC02 | Francci3_0926–Francci3_0931 | Type I iterative PKS | Unknown, same as FA03 | Unk |
| FC03 | Francci3_0987–Francci3_1000 | Type I iterative PKS | Halogenated lipid | Unk |
| FC04 | Francci3_1111–Francci3_1120 | PKS | Fragmented/nonproducing cluster? | Unk |
| FC05 | Francci3_1178 | Quinone "NRPS" | Unknown quinones | 12 |
| FC07 | Francci3_1926–Francci3_1934 | FAS/PKS | Cyclopropanated lipids | Unk |
| FC08 | Francci3_1967–Francci3_1993 | Hybrid PKS-NRPS | Modified glycine | Unk |
| FC11 | Francci3_2406 | Quinone "NRPS" | Unknown quinones | 12 |
| FC12 | Francci3_2442–Francci3_2466 | NRPS | Potential antibiotic | 13 |
| FC13 | Francci3_2596 | PKS (KS domain) | Fragmented/nonproducing cluster? | Unk |
| FC14 | Francci3_2845–Francci3_2867 | Type II PKS | Spore pigment, same as FA20 | Unk |
| FC15a | Francci3_2921–Francci3_2941 | Type I iterative PKS | PUFA | 5 |
| FC15b | Francci3_2981–Francci3_2987 | Type I PKS | Fragmented/nonproducing cluster? | Unk |
| FC17 | Francci3_4095–Francci3_4107 | Type II PKS | Octaketide antiobiotic | Unk |
| FC18 | Francci3_4124–Francci3_4155 | Incomplete type II PKS | None? Or tailoring for FC17? | Unk |
| FC19 | Francci3_4231 | Terpene cyclase | Geosmin | 21 |
| FC20 | Francci3_4330–Francci3_4335 | Terpene cyclase | Oxidized pentalenene | 22 |
| FC22 | Francci3_1383–Francci3_1398 | Terpene synthase | Carotenoids, same as FA25 | 20 |
| FC23 | Francci3_818–Francci3_834 | Terpene synthase | Hopanoids | 15-19 |
| FC24 | Francci3_4054–Francci3_4061 | Non-NRPS siderophore | Siderophore, same as FA23, FE06 | Unk |
| FC25 | Francci3_2983–Francci3_2987 | Ribosomal peptide | Microcin-like | Unk |
| FC26 | Francci3_4195–Francci3_4202 | Thiopeptide | Thiocillin-like | Unk |
| | | | | |
| *Frankia* sp. EAN1pec | | | | |
| FE01 | Franean1_3052–Franean1_3065 | NRPS | Tetrapeptide siderophore | 10 |
| FE03 | Franean1_2387–Franean1_2400 | Type II PKS | Spore pigment, same as FA20 | Unk |
| FE04 | Franean1_1665 | Quinone "NRPS" | Unknown quinones | 12 |
| FE06 | Franean1_655–Franean1_662 | Non-NRPS siderophore | Siderophore, same as FA23, FC24 | Unk |
| FE07 | Franean1_5933–Franean1_5952 | NRPS | Hexapeptide siderophore | 11 |
| FE08 | Franean1_5774–Franean1_5780 | Type I PKS | Unknown | Unk |
| FE09 | Franean1_5610–Franean1_5615 | Type I iterative PKS | Unknown, same as FA03 | Unk |
| FE10 | Franean1_5559 | Terpene cyclase | Geosmin | 21 |
| FE11 | Franean1_5372–Franean1_5375 | PKS (KS domain) | Fragmented cluster? | Unk |
| FE12 | Franean1_4821–Franean1_4849 | Type I PKS | Polyketide with AHBA starter | 8 |
| FE15 | Franean1_4393–Franean1_4396 | Type III PKS | Unknown, see also FA24 | Unk |
| FE16 | Franean1_4260–Franean1_4280 | Type I PKS | Fragmented/nonproducing cluster? | Unk |
| FE17 | Franean1_5590–Franean1_5618 | Type I iterative PKS | PUFA | 5 |
| FE18 | Franean1_3882–Franean1_3902 | Type I modular PKS | Specialized lipid | 3 |
| FE20 | Franean1_3607–Franean1_3623 | Type I iterative PKS | Aromatic compound | 6 |
| FE21 | Franean1_3454–Franean1_3497 | Type I PKS | Specialized lipid | 4 |
| FE22 | Franean1_3355–Franean1_3370 | Type I PKS | Mycocerosate-like lipids | 1 |
| FE23 | Franean1_3077–Franean1_3091 | Type I PKS | Fragmented/nonproducing cluster? | Unk |
| FE24 | Franean1_5691–Franean1_5718 | Terpene synthase | Hopanoids | 15-19 |
| FE25 | Franean1_5118–Franean1_5132 | Terpene synthase | Carotenoids, same as FA25 | 20 |

[a] Numbers correspond to predicted structures shown in Fig. 2. Unk, unknown.

homologous eight-gene NRPS-independent siderophore (6) biosynthetic locus (FA23, FC24, and FE06) for the assembly of an aerobactin-like siderophore. The *Frankia* genomes also carry isolated NRPS-encoding genes such as in FA05, FC05, and FE04 that may be involved in the biosynthesis of small quinone metabolites (compound 12).

CcI3 and ACN contain giant NRPS gene clusters (FC12 and FA08) associated with the biosynthesis of cyclic peptides. Clus-
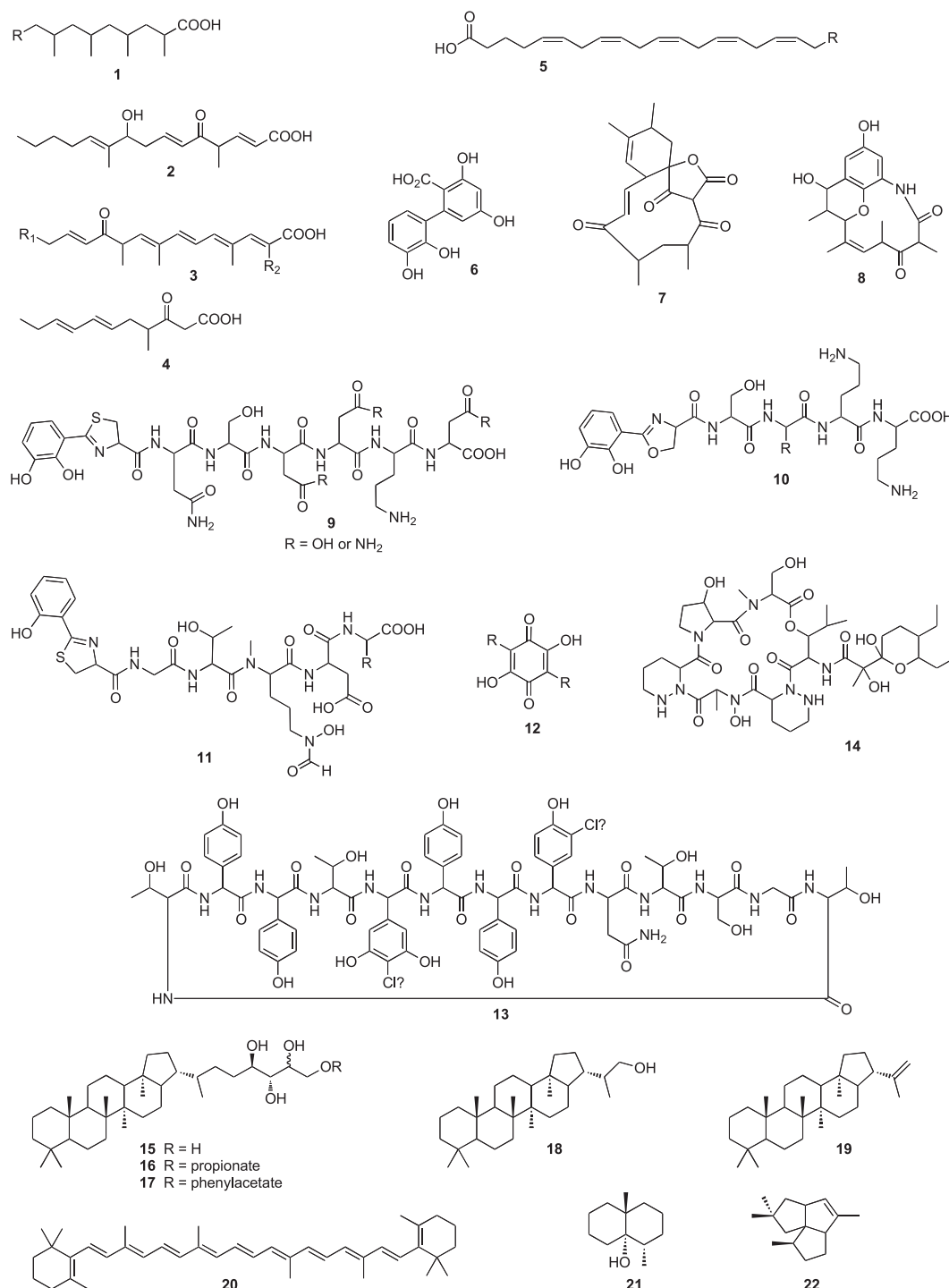
FIG. 2. Putative *Frankia* chemical structures based on bioinformatic analysis of *Frankia* biosynthetic gene clusters. Compound numbers correlate with the "Predicted structure" data in Table 1.

ter FC12 is expected to produce a unique, halogenated tridecapeptide (compound 13), which may have antibacterial properties based upon its predicted structural resemblance to existing antibiotics of the vancomycin group (26). In addition to the large, 13-module NRPS system, we identified gene cassettes involved in the biosynthesis of the vancomycin-containing nonproteinogenic amino acids 4-hydroxyphenylglycine and 3,5-dihydroxyphenylglycine. While many of the FC12 gene products, including a flavin adenine dinucleotide (FAD)-dependent halogenase that putatively chlorinates phenol residues, resemble those of the vancomycin/teichoplanin class of antibiotics, the cytochrome P450 coupling enzymes that are required for the cross-coupling of vancomycin's aromatic rings are absent (57). As a consequence, the product of FC12 (com-

TABLE 2. Mass spectrometry of *Frankia* sp. EAN1pec and *Frankia* sp. CcI3

| Species and gene cluster | Gene | Protein | Putative protein function | No. of peptides | No. of spectra | % coverage |
|---|---|---|---|---|---|---|
| *Frankia* sp. EAN1pec | | | | | | |
| FE01 | Franean1_3063 | 158110278 | Amino acid adenylation domain | 1 | 2 | 0.2 |
| FE03 | Franean1_2391 | 158109626 | Cyclase/dehydrase | 2 | 2 | 26.8 |
| | Franean1_2396 | 158109631 | Antibiotic biosynthesis monooxygenase | 3 | 3 | 43.9 |
| FE07 | Franean1_5939 | 158113086 | Periplasmic binding protein | 7 | 16 | 37.6 |
| | Franean1_5945 | 158113092 | ABC transporter related | 2 | 2 | 6 |
| FE10 | Franean1_5558 | 158112715 | Cyclic nucleotide-binding domain protein | 38 | 154 | 71.9 |
| | Franean1_5559 | 158112716 | Terpene synthase metal-binding domain protein | 6 | 6 | 11.2 |
| FE16 | Franean1_4274 | 158111461 | Isocitrate dehydrogenase NADP-dependent | 24 | 59 | 35 |
| FE17 | Franean1_5592 | 158112747 | Serine/threonine protein kinase | 6 | 8 | 16.9 |
| | Franean1_5595 | 158112750 | FHA modulated ABC efflux pump with fused ATPase and integral membrane subunits | 8 | 13 | 13.6 |
| | Franean1_5596 | 158112751 | Hypothetical protein Franean1_5596 | 5 | 7 | 18 |
| | Franean1_5606 | 158112761 | 6-Phosphogluconate dehydrogenase, decarboxylating | 2 | 2 | 7.6 |
| | Franean1_5616 | 158112770 | Transcriptional regulator LuxR family | 2 | 4 | 7.2 |
| FE18 | Franean1_3892 | 158111085 | Conserved hypothetical protein | 23 | 32 | 35.7 |
| | Franean1_3902 | 158111095 | Conserved hypothetical protein | 2 | 10 | 20 |
| FE21 | Franean1_3497 | 158110702 | Superoxide dismutase | 4 | 6 | 30.5 |
| FE22 | Franean1_3364 | 158110569 | Endothelin-converting enzyme 1 | 2 | 2 | 3.5 |
| FE24 | Franean1_5711 | 158112864 | Radical SAM domain protein | 2 | 3 | 8.4 |
| | Franean1_5715 | 158112868 | Amine oxidase | 2 | 3 | 7.8 |
| | Franean1_5717 | 158112870 | Squalene/phytoene synthase | 2 | 2 | 6.9 |
| *Frankia* sp. CcI3 | | | | | | |
| FC01 | Francci3_364 | 86565942 | Hypothetical protein Francci3_0364 | 2 | 7 | 40 |
| FC03 | Francci3_990 | 86566564 | 3-Oxoacyl-[acyl-carrier-protein] synthase III | 8 | 8 | 11.4 |
| | Francci3_991 | 86566565 | Acyl transferase region | 8 | 15 | 7.9 |
| | Francci3_993 | 86566567 | FAD dependent oxidoreductase | 1 | 2 | 1.3 |
| | Francci3_999 | 86566573 | Crotonyl-CoA reductase | 3 | 3 | 7 |
| FC05 | Francci3_1179 | 86566749 | Heat shock protein Hsp20 | 10 | 27 | 37.4 |
| FC08 | Francci3_1985 | 86567550 | Hypothetical protein Francci3_1985 | 2 | 3 | 25 |
| FC12 | Francci3_2450 | 86568008 | Amino acid adenylation | 16 | 22 | 3.2 |
| | Francci3_2452 | 86568010 | Aminotransferase class I and II | 1 | 2 | 3.4 |
| | Francci3_2454 | 86568012 | 4-Hydroxyphenylpyruvate dioxygenase | 30 | 86 | 31.8 |
| | Francci3_2459 | 86568017 | Amino acid adenylation | 4 | 5 | 1.3 |
| | Francci3_2461 | 86568019 | Amino acid adenylation | 6 | 11 | 2 |
| FC14 | Francci3_2861 | 86568410 | Enoyl-CoA hydratase | 2 | 3 | 8.6 |
| FC15a | Francci3_2925 | 86568474 | HpcH/HpaI aldolase | 2 | 2 | 8.4 |
| FC19 | Francci3_4230 | 86569767 | Cyclic nucleotide-binding domain protein | 64 | 194 | 61.7 |
| | Francci3_4231 | 86569768 | Terpene synthase, metal-binding | 9 | 11 | 11.7 |
| FC23 | Francci3_833 | 86566408 | Urease beta subunit | 1 | 2 | 14.3 |

pound 13) is predicted to resemble cyclic peptide antibiotics related to ramoplanin A2 and enduracidin (59). Cluster FA08 codes for the biosynthesis of a hybrid PKS/NRPS-derived molecule (compound 14) related in chemical structure to the polyoxypeptin family of potent apoptosis-inducing *N*-acyl cyclic hexadepsipeptides (56).

**Gene clusters associated with terpenoid biosynthesis.** Hopanoids are important lipid components of the *Frankia* vesicle envelope, where they account for up to 87% of the total lipids (11, 38). Predominant *Frankia* hopanoids identified in CcI3 and other strains include bacteriohopanetetrols (compounds 15 to 17) and moretan-29-ol (compound 18), which are assembled from hopene (compound 19) by squalene-hopene cyclase (Shc) (2, 18). Their production is governed by a homologous *shc*-containing gene cluster (FA26, FC23, and FE24) in the three sequenced *Frankia* genomes related in structure and organization to those in *S. coelicolor* A3(2), *Bradyrhizobium japonicum* USDA110, and *Zymomonas mobilis* ZM4, which produce related hopanoid lipids (39, 42). Conversion of bacteriohopanetetrol (compound 15) to its novel 35-*O*-phenylace-

tate monoester (compound 17) (46) may be assisted by a phenylacetate-degrading enzyme derived from FA26 and FC23. All three *Frankia* hopanoid gene clusters uniquely harbor a radical *S*-adenosylmethionine (SAM)-dependent methyltransferase that may be involved in production of methylated derivatives, which are components of the $N_2$-fixing bacterium *B. japonicum* (12) and other bacteria (45).

Sequence analysis of ACN, CcI3, and EAN also revealed conserved terpenoid pathways to carotenoids (FA25, FC22, and FE25) such as β-carotene (compound 20) and zeaxanthin diglucoside and the odorous terpenoid geosmin (FA28, FC19, and FE10; compound 21), which are common streptomycete metabolites (39). Additional terpenoid natural products may be produced in ACN and CcI3 via FA27 and FC20, respectively. These biosynthetic loci contain terpene cyclase genes that may code for the assembly of sesquiterpenoids derived from the tricyclic pentalenene hydrocarbon (compound 22), which in *S. avermitilis* gives rise to the pentalenolactone family of antibiotics (51). In all cases, *Frankia* bacteria exclusively employ the methylerythritol phosphate pathway to the iso-
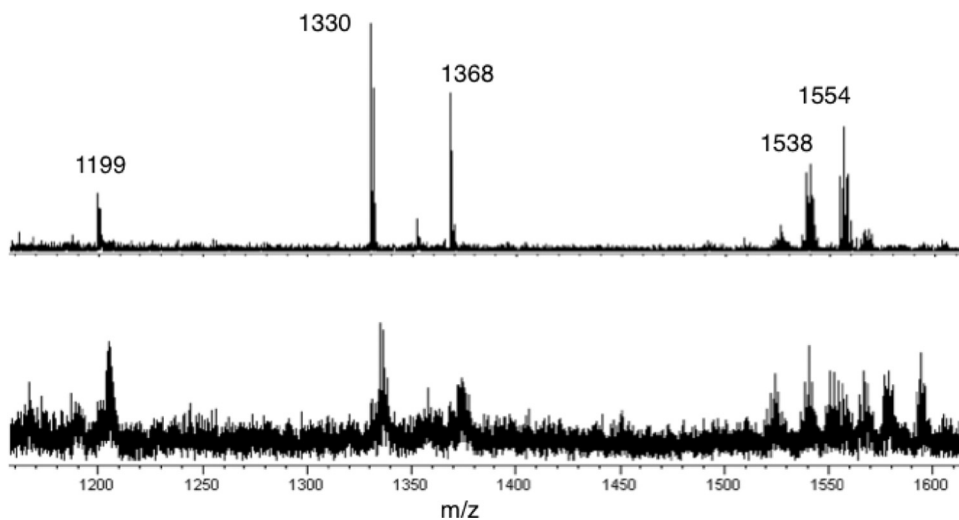
FIG. 3. Intact-cell MALDI-TOF analysis of *Frankia* sp. CcI3. Top, IC MALDI revealing prominent peptides ranging from 1,199 to 1,554 Da. Bottom, IC MALDI of *Frankia* sp. CcI3 grown on $^{15}$N-labeled medium depicting isotope shifts that roughly equate to the number of amino acid residues.

prenoid building blocks isopentenyl diphosphate and its isomer dimethylallyl diphosphate as observed in most actinomycetes (39), although all three *Frankia* strains possess more than one copy of 4-hydroxy-3-methylbut-2-enyl diphosphate reductase (*ispH*) and 2-*C*-methyl-*d*-erythritol 4-phosphate cytidylyltransferase (*ispD*).

**Other *Frankia* secondary metabolic pathway gene clusters.** Additional biosynthetic gene clusters are predicted for the assembly of bacteriocin, aminocyclitol, and phosphonate natural products. Ribosomally encoded peptides related to the *Escherichia coli* lasso peptide microcin J25 are predicted to occur in ACN (FA30) and CcI3 (FC25) (48). A highly modified thiopeptide related to the *Bacillus subtilis* antibiotic thiocillin (13) is putatively produced in CcI3 (FC26). *Frankia alni* ACN contains two additional biosynthetic gene clusters (FA31 and FA29) related to cetoniacytone A, an aminocyclitol antitumor agent from the endosymbiont *Actinomyces* sp. strain Lu 9419 (58), and the antibiotic and herbicidal phosphinothricin tripeptide from *Streptomyces viridochromogenes* (49). In cluster FA29, a conserved set of six genes encodes the biosynthesis of the phosphinothricin intermediate phosphonoformate. Additional biosynthetic genes in FA29 suggest that pathways diverge to give an unknown phosphonate-containing secondary metabolite in ACN (39). Since natural phosphonates exhibit herbicidal properties, the production of such a molecule in a plant symbiont is an intriguing possibility.

**Proteomic evidence of gene cluster expression in CcI3 and EAN.** Further insight into the gene clusters that may actively produce natural products under laboratory culture conditions was explored by proteomic analysis of strains EAN and CcI3. Mass spectrometry analysis uncovered 10 candidate expressed gene clusters in EAN that contain at least one protein hit from at least two spectra (Table 2). Of the 10 candidates, 6 (FE03, FE07, FE10, FE17, FE18, and FE24) had at least two protein hits corresponding to adjacent encoding genes from each gene set. Conversely, nine candidate expressed gene clusters were detected in *Frankia* sp. CcI3, of which three (FC03, FC12, and

FC19) contained two or more protein hits with two peptide identifications per protein. Furthermore, other transcriptome and proteome studies show expression of some of these biosynthetic clusters (1, 3, 5, 33, 34, 44). These data together indicate that *Frankia* has both the genetic capacity and the biosynthetic capacity to produce secondary metabolites.

Intact-cell MALDI-TOF (IC MALDI-TOF) mass spectrometry, which can be used to observe natural products from organisms with minimal work-up (20), was next employed to probe strain CcI3. IC MALDI-TOF mass spectrometry showed several significant signals in the mass range of NRPS-derived secondary metabolites (Fig. 3). The ions at 1,538 and 1,554 Da displayed a halogen isotopic signature, as the +2-Da isotopes are larger than the monoisotopic mass and are within the expected range of nonribosomal peptide compound 13. In addition, several ions above 1 kDa were uniquely observed in *Frankia* strain CcI3. To provide further support for the presence of unique peptides, the CcI3 bacterium was grown in the presence of $^{15}$N-labeled NH$_4$Cl. In this case, the ions at 1,199, 1,330, 1,368, 1,538, and 1,554 Da shifted, and their isotopic distributions broadened, indicating incorporation of $^{15}$N. The ions at 1,538 and 1,554 Da shifted 13 Da, consistent with the prediction that the product of FC12 is a tridecapeptide, such as compound 13.

**Summary.** Our results here reveal the significant biosynthetic potential of the genus *Frankia* in the production of novel gene-encoded small molecules. Across the three strains examined, we found nearly all of the common classes of secondary metabolic biosynthetic pathways. At the time of writing, eight additional *Frankia* genome projects are under way: those for *Frankia* strain EuI1c (GenBank accession no. CP002299), *Frankia* strain EUN1f, *Frankia* strain QA3, *Frankia* strain BCU110501, *Frankia* strain BMG5.12, *Frankia* strain CN3, *Frankia* strain DC12, and the *Frankia* symbiont from *Datisca glomerata*. On cursory examination of available data, each shows genetic biosynthetic potential comparable to that of the strains analyzed here. The illumination of the biosynthetic

potential of *Frankia* should have a significant impact on the study of host-microbe interactions. Symbiotic interactions between *Frankia* and its host plants are not well understood at a molecular level. Although there are aspects common to other plant-microbe interactions, including signaling pathways shared between fungal and bacterial root endosymbioses in the actinorhizal plant *Casuarina* and in legumes (22), the actual signaling molecules have not been identified for the actinorhizal associations. The absence of common *nod* genes in the *Frankia* genomes indicates the use of alternative signaling molecules. The predicted structures in this study provide tempting targets as cell signaling molecules during the establishment and development of the symbiotic association.

## ACKNOWLEDGMENTS

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