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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 determination 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

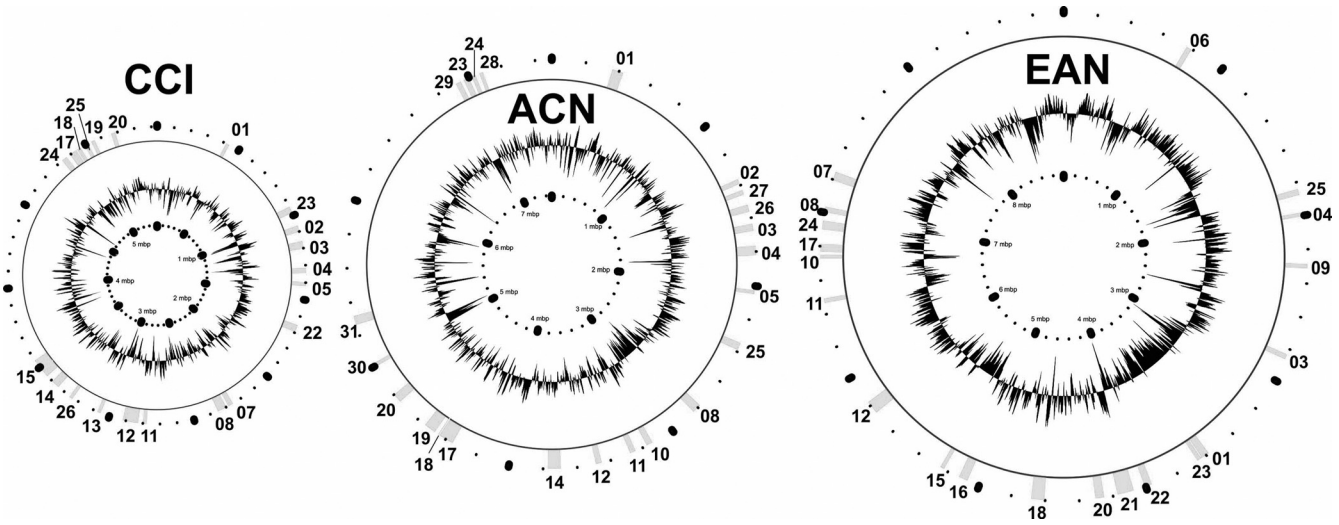

FIG. 1. Circular chromosomes of *Frankia* sp. HFPCcI3 (CCI), *Frankia* sp. ACN14a (ACN), and *Frankia* sp. EAN1pec (EAN), oriented to the *dnaA* gene. The inner ring indicates deviation of GC content from the genomic average. The outside outer ring shows the locations of secondary metabolic gene clusters 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-

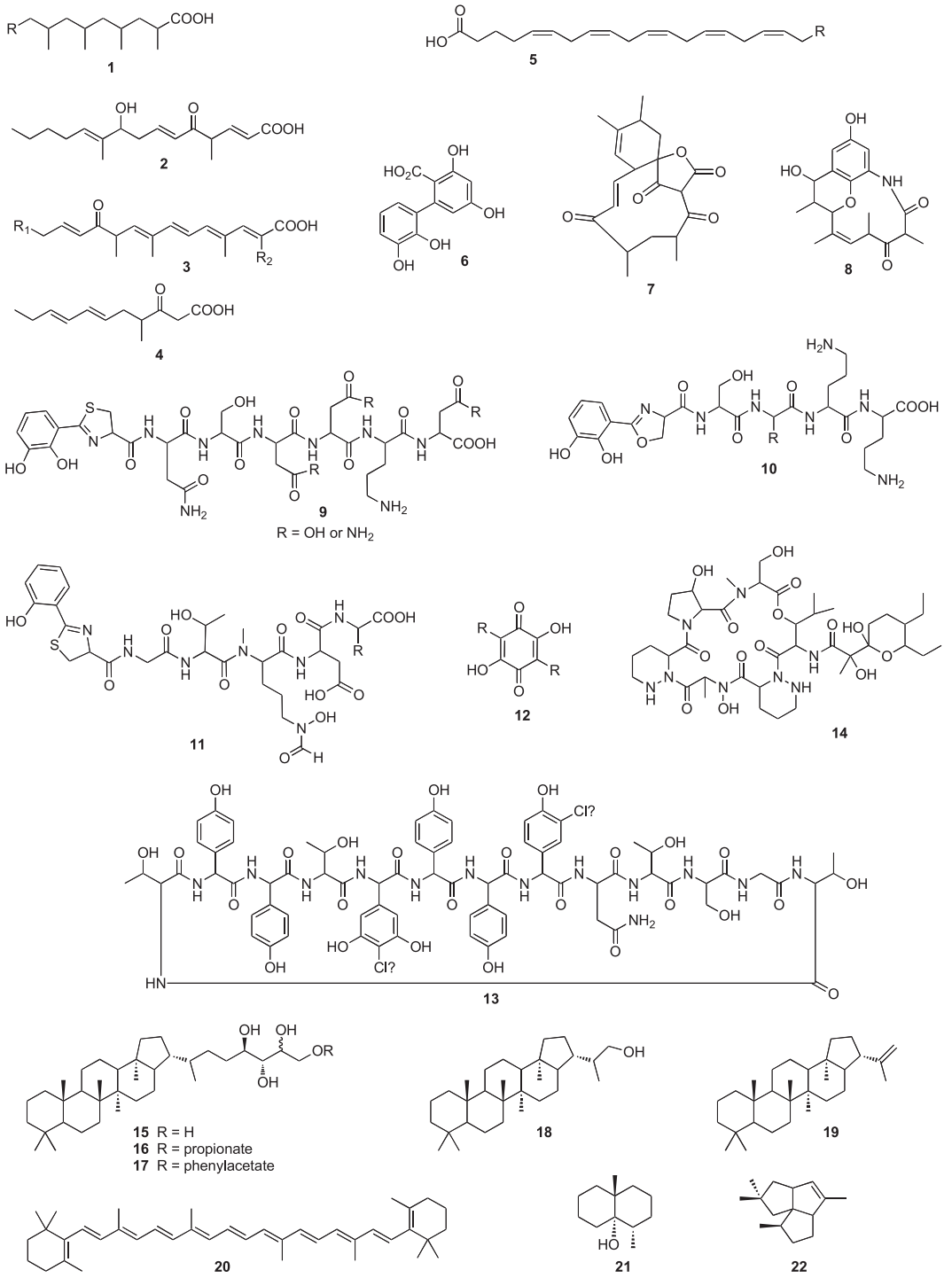

FIG. 2. Putative *Frankia* chemical structures based on bioinformatic analysis of *Frankia* biosynthetic gene clusters. Compound numbers correlate with the "Predicted structure" data in Table 1.

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*-phenylacetate 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-

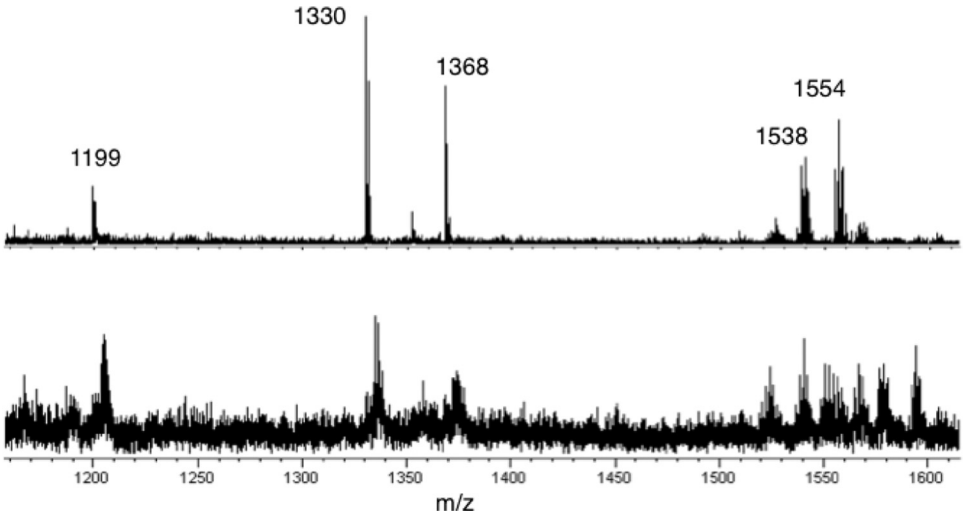

FIG. 3. Intact-cell MALDI-TOF analysis of *Frankia* sp. CcI3. Top, IC MALDI revealing prominent peptides ranging from 1,199 to 1,554 Da. Bottom, IC MALDI of *Frankia* sp. CcI3 grown on ^{15}N-labeled medium depicting isotope shifts that roughly equate to the number of amino acid residues.

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_4Cl. 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.

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