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Diminished Exoproteome of *Frankia* spp. in Culture and Symbiosis▽†

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***Frankia* species are the most geographically widespread gram-positive plant symbionts, carrying out N_2 fixation in root nodules of trees and woody shrubs called actinorhizal plants. Taking advantage of the sequencing of three *Frankia* genomes, proteomics techniques were used to investigate the population of extracellular proteins (the exoproteome) from *Frankia*, some of which potentially mediate host-microbe interactions. Initial two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of culture supernatants indicated that cytoplasmic proteins appeared in supernatants as cells aged, likely because older hyphae lyse in this slow-growing filamentous actinomycete. Using liquid chromatography coupled to tandem mass spectrometry to identify peptides, 38 proteins were identified in the culture supernatant of *Frankia* sp. strain CcI3, but only three had predicted export signal peptides. In symbiotic cells, 42 signal peptide-containing proteins were detected from strain CcI3 in *Casuarina cunninghamiana* and *Casuarina glauca* root nodules, while 73 and 53 putative secreted proteins containing signal peptides were identified from *Frankia* strains in field-collected root nodules of *Alnus incana* and *Elaeagnus angustifolia*, respectively. Solute-binding proteins were the most commonly identified secreted proteins in symbiosis, particularly those predicted to bind branched-chain amino acids and peptides. These direct proteomics results complement a previous bioinformatics study that predicted few secreted hydrolytic enzymes in the *Frankia* proteome and provide direct evidence that the symbiosis succeeds partly, if not largely, because of a benign relationship.**

The *Frankia*-actinorhizal plant symbiosis is widespread in nature, providing fixed nitrogen to nearly 200 known species of plants collectively distributed on every continent, except Antarctica, and in most climate zones (5). Actinorhizal plants are pioneer species that add nitrogen and organic material to nutrient-poor or new soils. Some species are grown commercially for timber and windbreaks, such as *Alnus* (alder) and *Casuarina* trees. When infected with *Frankia*, root nodules that appear as repeatedly branching truncated lateral roots are induced.

Frankia spp. are N_2-fixing, filamentous members of the *Actinobacteria*. Strains are phenotypically diverse, colonizing distinct subgroups of plants. Three sequenced genomes (strains CcI3, ACN14a, and EAN1pec) span a range of sizes from 5.4 Mb to 9 Mb (26). Genome sequencing has allowed the use of high-throughput technologies, such as proteomics and transcriptomics, to discover actively expressed genes and shape conclusions about *Frankia* physiology in culture and symbiosis.

Extracellular and surface-associated proteins are of particular interest in symbioses, as the interface between the bacterium and plant is the first zone of contact, where proteins involved in molecular recognition, polymer degradation, solute binding, or defense may be localized. In the rhizobium-legume symbiosis, secreted proteins implicated in plant interactions include some that sense plant flavonoids, modify surface polysaccharides, bind calcium, or otherwise directly affect the biology of the host, such as the "Nops" (nodulation outer proteins), which are injected into host cells by type III or type IV secretion systems in some strains (8, 11). Essentially nothing is known about bacterial proteins involved in molding the actinorhizal symbiosis on plant roots.

Unlike related soil actinobacteria, such as *Streptomyces* spp., that secrete an array of degradative enzymes, *Frankia* strains have been predicted to secrete comparatively few proteins, according to an extensive bioinformatics study that predicted frankial secreted proteomes based on consensus signal peptides and transmembrane domains (12, 22). While several *Streptomyces* species are predicted to secrete more than 100 hydrolases, the *Frankia* secreted proteomes have only 10 to 20, and these are primarily lipases, esterases, and proteases, rather than putative polysaccharide-degrading enzymes (22). This observation raises the hypothesis that frankiae are successful symbionts at least partly because they present an innocuous visage to the plant host.

The culture supernatant of *Frankia alni* strain ACN14a, an *Alnus* symbiont, was recently investigated, using individual protein spots excised from two-dimensional (2D) sodium dodecyl sulfate (SDS)-polyacrylamide gels (1). Several glycolytic and tricarboxylic acid cycle enzymes, among other cytoplasmic proteins, were found in the extracellular fraction, but few signal peptide-containing proteins were detected (1), raising the possibility that cell lysis was responsible for many of the proteins detected. A comparison with the symbiotic state was not done at that time.

To provide a more complete overview of secreted proteins in light of our previous bioinformatics study (22), we investigated both free-living and symbiotic cells of another *Frankia* strain, the *Casuarina* symbiont CcI3, using 2D SDS-polyacrylamide gel electrophoresis (PAGE) and liquid chromatography cou-

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pled to tandem mass spectrometry (LC MS/MS) of trypsin-digested protein samples.

MATERIALS AND METHODS

Bacterial strains and culture. *Frankia* sp. strain CcI3, originally isolated from greenhouse-grown *Casuarina cunninghamiana* (38), was grown in liquid *Frankia* defined minimal medium (FDM) with 0.5% pyruvate in stationary 100-ml cultures, in 250-ml screw-cap flasks, at 30°C (http://web.uconn.edu/mcbstaff/benson/Frankia/FDM.htm). Every 3 or 4 days, cells were collected by centrifugation, homogenized with a Dounce-type tissue homogenizer to break up mycelia, and washed twice in fresh medium before transfer. Cells corresponding to ~0.5 mg total protein were transferred into each flask. Strain CpI1, originally isolated from *Comptonia peregrina* (7), was cultured similarly, but in FDM containing 0.5% succinate, with shaking at 125 rpm at 30°C.

Preparation of samples for 2D gel electrophoresis. For proteins in the culture supernatant, cells were removed by centrifugation at 10,000 × *g*, and the supernatant was clarified by vacuum filtration through a 0.22-μm filter. Protein from the filtered supernatant (100 ml) was concentrated by dialysis against polyethylene glycol (PEG 8000; Sigma, St. Louis, MO) to a final volume of 1 to 5 ml. Cytoplasmic protein was obtained from ~0.5 ml of packed cells, resuspended in FDM, and sonicated (4 min at 40% duty cycle). After sonication, the suspension was centrifuged at 16,000 × *g*, and the supernatant was saved as the cytoplasmic sample.

Protein was quantified from culture supernatants and cytoplasmic extracts with the bicinchoninic acid (BCA) assay (Pierce Chemical Co., Rockford, IL) (32). To concentrate the samples, proteins were precipitated with 10% trichloroacetic acid (TCA; Sigma Chemical Co., St. Louis, MO), either at 4°C overnight or at −20°C for several hours. The precipitate was centrifuged at 11,000 × *g* for 30 min, washed twice with ice-cold acetone, and collected by centrifugation at 16,000 × *g* for 10 min.

For 2D SDS-PAGE, TCA-precipitated protein pellets were dissolved in rehydration/sample buffer (Bio-Rad, Hercules, CA), applied to a 7-cm immobilized-pH-gradient strip (pH 4 to 7), and then subjected to isoelectric focusing using the conditions recommended by the manufacturer (Bio-Rad). Protein loading on gels was empirically determined to give similar spot intensities on both cytoplasmic and supernatant gels. Generally, around 20 to 30 μg of protein was loaded. After equilibration of the immobilized-pH-gradient strip according to the manufacturer's instructions, the second dimension was run on an 11% polyacrylamide gel, at 200 V for 45 min. Gels were stained using a silver staining procedure based on the study by Blum et al. (6), as follows: fixation for 10 min in 40% methanol-13.5% formalin, with two 5-min rinses in water; addition of 0.02% $Na_2S_2O_3$ for 1 min; 0.1% $AgNO_3$ staining for 10 min; developing with a solution of 3% Na_2CO_3-0.05% formalin-0.000016% $Na_2S_2O_3$; stopping development with 2.3 M citric acid.

Preparation of root nodule protein samples. *Casuarina cunninghamiana* and *Casuarina glauca* seeds were washed with 20% household bleach and germinated on water agar or in sterile sand. Seedlings (2 to 4 weeks old) were inoculated with *Frankia* sp. strain CcI3, by soaking roots for several hours in 10 mM phosphate buffer containing bacterial cells. Plants were grown under natural sunlight in a greenhouse and transferred to larger pots containing sterile sand as needed. Three root nodule samples were collected: two from *C. cunninghamiana* (9 and 11 months old) and one from *C. glauca* (4 years old). Nodules were dissected in ice-cold TEA buffer (10 mM Tris-HCl, 1 mM Na_2EDTA, 20 mM sodium ascorbate [pH 7.6]) (Sigma, St. Louis, MO). The younger, 1- to 2-mm tips of nodule lobes were collected to enrich for metabolically active bacterial cells, and the root epidermis was gently peeled using a scalpel and dissecting forceps while being viewed through a dissecting microscope. The infected cortical cells were then scraped from the vascular tissue and placed in a separate microcentrifuge tube. The remains of 150 peeled root nodule lobes per sample were macerated in TEA buffer, using a Dounce-type tissue homogenizer. The nodule cell material was washed several times in TEA buffer, followed by six washes in 50 mM Tris-Cl (pH 6.8) to remove plant phenolics. The infected plant cells were sonicated for 5 to 10 min (50% duty cycle) in 50 mM Tris-Cl (pH 6.8) plus 5 mM Na_2EDTA, to release bacterial proteins. Insoluble material was pelleted, and the soluble protein was quantified using the Pierce BCA assay (32). The resulting protein extract was enriched in *Frankia* proteins from all cellular compartments, along with plant proteins that were associated with the infected cells. The resulting peptides were binned using an informatics approach into plant and bacterial fractions and further into those that are in the predicted *Frankia* exoproteome. We report here only those proteins predicted to form part of the exoproteome.

Field-collected root nodules of *Alnus incana* subsp. *rugosa* and *Elaeagnus angustifolia* were sampled from 2- to 3-foot-tall shrubs growing in roadside lots off Route 32 (Willington, CT) and Route 195 (Willington, CT), respectively, in June and July 2008. Nodules were dissected within 2 hours of collection in ice-cold TEA buffer and prepared as described above. Roughly 100 root nodule lobes were harvested per plant.

Preparation of samples for LC MS/MS analysis. Supernatants from four separate CcI3 cultures between 2 and 5 days old were analyzed; one was analyzed from strain CpI1 (3 days old). Instead of PEG precipitation, supernatants were lyophilized, frozen at −80°C, and then thawed and resuspended in 5 to 10 ml water prior to TCA precipitation (described above). Root nodule protein extracts, containing 0.35 to 1 mg protein as measured by the BCA assay, were also TCA precipitated.

Precipitated protein pellets were dried briefly (5 min) and dissolved in 8 M urea-0.4 M NH_4HCO_3 (pH 7.5 to 8.5). Disulfide bonds were reduced by adding 7.5 mM of dithiothreitol and incubating for 20 min at 37°C. Iodoacetamide was added to 15 mM to block sulfhydryl groups, and the solution was incubated at room temperature for 20 min in the dark. The solution was diluted fourfold in water, and sequencing-grade trypsin was added according to the manufacturer's instructions (Promega, Madison, WI). Trypsin digestion was carried out at 37°C for 18 to 24 h, and digested samples were frozen at −80°C.

LC MS/MS of culture supernatant samples. LC MS/MS analysis was performed on a Waters capillary LC coupled to a Micromass Q-Tof Ultima mass spectrometer (Waters, Milford, MA). Trypsin-digested peptides were dried and resuspended in 3 μl 70% formic acid, and 0.1% trifluoroacetic acid (TFA) was added to bring the volume to 10 μl. Samples (5 μl) were injected into a 0.1-mm by 150-mm Atlantis C_{18} column (Waters) and run at 500 nl/minute. High-performance liquid chromatography was started with 95% buffer A (98% water, 2% acetonitrile, 0.1% acetic acid, and 0.01% TFA) and 5% buffer B (20% water, 80% acetonitrile, 0.09% acetic acid, and 0.01% TFA). A linear gradient was run with increasing amounts of buffer B, as follows: 5% buffer B for 3 min, 37% buffer B for 43 min, 75% buffer B for 75 min, and 95% buffer B for 85 min. Eluted peptides were ionized by electrospray, peptide masses were measured, and doubly or triply charged peptides were fragmented. To enhance fragmentation, a collision energy ramp corresponding to mass and charge state was established. The mass spectrometer was run in data-dependent acquisition mode, alternating between MS and MS/MS modes when the total ion current exceeded a threshold of 1.5 counts/second. MS/MS spectra were analyzed with the Mascot database search algorithm (Matrix Science, Boston, MA) (30).

LC MS/MS of root nodule extracts with multidimensional protein identification technology. The trypsin digests from the root nodule protein samples were fractionated by strong cation exchange (SCX) chromatography on an Applied Biosystems Vision workstation. Prior to SCX, the sample was acidified with 2 μl of 1 M phosphoric acid. A linear gradient was run for 118 min on a 2.1-mm by 200-mm PolySulfoethyl A column (PolyLC Inc.). The gradient was from 99.3% to 2.0% of buffer A (10 mM potassium phosphate, 25% acetonitrile [pH 3.0]), with increasing concentrations of buffer B (same as buffer A but with 1 M potassium chloride added). Fractions were dried, dissolved in 5 μl of 70% formic acid, and diluted to 15 μl in 0.1% TFA. For each of the three *Casuarina* nodule replicate samples, 10 pooled fractions were used. For the *A. incana* and *E. angustifolia* samples (one each), 20 pooled fractions were analyzed.

LC MS/MS of pooled SCX fractions was performed with a nanoAcquity ultra-performance LC system (Waters) coupled to an LTQ Orbitrap mass spectrometer (Thermo Fisher Scientific). LC columns were a Waters Symmetry C_{18} (180-μm by 20-mm) trap column and a 1.7-μm particle nanoAcquity ultra-performance LC column (75 μm × 250 mm). Initial conditions were 95% buffer A (100% water, 0.1% formic acid) and 5% buffer B (100% acetonitrile, 0.075% formic acid). A 51-min linear gradient was run with increasing amounts of buffer B, as follows: 5% buffer B starting, up to 50% buffer B at 50 min, and 85% buffer B at 51 min. The LTQ Orbitrap performs one microscan to acquire the MS, and then eight data-dependent MS/MS acquisitions are carried out in the ion trap. MS/MS spectra were analyzed with the Mascot database search algorithm (30).

Identification of supernatant protein spots by MS/MS. Three of the darkest protein spots detected in CcI3 supernatants were selected for identification. Using a 2D gel of protein concentrated from four combined flasks of 4-day-old supernatants (400 ml total), Coomassie blue-stained gel spots were excised, cut into small pieces, and added to 1.5-ml Eppendorf tubes (prewashed with 500 μl 0.1% TFA-60% CH_3CN). Gel pieces were washed with 250 μl of 50% H_2O-50% acetonitrile for 5 min. After the wash solution was removed, 250 μl of 50% CH_3CN-50 mM NH_4HCO_3 was added, and samples were washed for 30 min at room temperature on a tilt table. Another 30-min wash was performed, using 50% CH_3CN-10 mM NH_4HCO_3 instead. Gel pieces were dried with a Speedvac, and 0.1 μg of modified trypsin (Promega, Madison, WI) was added per 15 mm^3 of gel in 15 μl 10 mM NH_4HCO_3 to all samples. After 5 to 10 min, an additional 20 μl 10 mM NH_4HCO_3 was added. The reaction mixtures were incubated at

37°C for 24 h, and then stored at −80°C prior to MS/MS analysis, which was performed on a Micromass Q-Tof Ultima mass spectrometer (Waters, Milford, MA) as described above for LC MS/MS of culture supernatants.

Protein identification with Mascot program. MS/MS spectra were converted into Mascot-compatible files by using the Mascot distiller program, which combines MS/MS scans from the same precursor ion. The program selects +2 and +3 ions with a signal-to-noise ratio of at least 1.2 and creates a peak list, which was searched by the Mascot algorithm against one or more *Frankia* genomes. All CcI3 protein samples (and the CpI1 supernatant sample) were searched versus the strain CcI3 genome, while the data from field-collected nodules were searched against the CcI3, ACN14a, and EAN1pec genomes. The FASTA amino acid sequences of the three *Frankia* genomes (CcI3, NCBI reference sequence [RefSeq] NC_007777; ACN14a, NCBI RefSeq NC_008278; EAN1pec, NCBI RefSeq NC_009921) were obtained from the NCBI RefSeq FTP site (ftp://ftp.ncbi.nih.gov/genomes/Bacteria).

Mascot specifications included a peptide tolerance window of ±0.6 Da, an MS/MS fragment tolerance of ±0.4 Da, peptide charges of +2 or +3, allowance of one missed cleavage by trypsin, and inclusion of amino acid modifications, such as methionine oxidation. Peptides are assigned a probability-based ion score, equal to $-10 \times \log_{10}P$, where P is the absolute probability that a match (between the observed peak lists and the theoretical peak lists in the database) is random. The probability of a random match depends on the size of the database, which is defined as the number of theoretical masses that fall within the peptide tolerance window provided. In this analysis, the threshold scores for significant peptide matches ($P < 0.05$) were assigned by Mascot as follows: ion scores of >15 were significant when using the CcI3 genome as a database, while scores of >16 were significant using the other two *Frankia* genomes. We considered a protein to be identified by Mascot if two or more unique peptides matched the protein. Mascot also assigns a nonprobabalistic protein score, derived from the individual peptide matches and the total number of queries. Mascot protein scores and peptide sequences identified in this study are provided in the supplemental material.

RESULTS AND DISCUSSION

2D SDS-PAGE. As a first step, we visualized proteins secreted from growing CcI3 cells by using a traditional 2D SDS-PAGE approach with cultures of various ages. *Frankia* cells grow slowly by hyphal tip extension rather than binary division, conferring a pseudo-linear, rather than exponential, mode of growth, without a clear stationary phase. Preliminary experiments indicated that frequent transfer (every 3 to 4 days) of washed hyphae, fragmented by homogenization, produces the most-uniform culture. Many *Frankia* studies use cells cultured for 10 days or more between transfer that contain a pronounced diversity of cells, including growing hyphal tips, nongrowing cells, and developing sporangia (4). Such cultures generally include an abundance of empty hyphae, indicating cell lysis (15).

Proteins in the supernatant of young, 3- or 4-day-old *Frankia* cells were visualized by 2D SDS-PAGE. When using carefully washed cells to start cultures, extracellular proteins concentrated from 100 ml of 3-day-old culture supernatants were at the limits of detection of silver-stained 2D gels, with fewer than 20 faint spots seen (not shown). When a greater volume (400 ml) was concentrated from 4-day-old cultures, roughly 40 spots could be visualized by Coomassie staining (Fig. 1A). Most of the protein spots from the supernatant were also found in the cytoplasmic fraction (Fig. 1B). This slow accumulation of extracellular proteins that overlap with cytoplasmic protein spots strongly suggests that cell lysis accounts for many of the proteins in the culture medium.

Three of the more abundant proteins were cut from the gels and identified by MS/MS (Fig. 1). Spots 1 and 2 were identified as small stress proteins, and were also present in the supernatants of strains CpI1 and ACN14a (gi 86738980 and gi 86742358) (Table 1). Homologs of these proteins were upregulated in several *Frankia* strains and *Streptomyces coelicolor* upon exposure to plant extracts (2, 13, 19). Their presence in the supernatant cannot be ascribed to specific secretion mechanisms, since neither has an identifiable signal peptide. Spot 3 is annotated as a phosphoesterase or a phosphatidylinositol-3-phosphate phosphatase, and contains a putative secretion signal sequence predicted by SignalP (3). The protein was also detected by one peptide in *Casuarina* nodules. Its gene is part of an operon containing several glycosyl transferase genes, suggesting a cell surface-related function. Spot 3 was the only major spot not visible in the cytoplasmic extract 2D gel (Fig. 1), an observation supporting its extracellular localization.

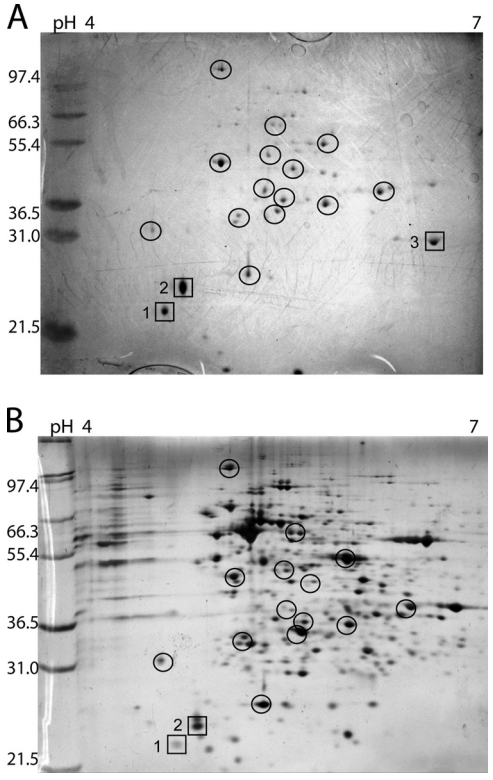

FIG. 1. (A) 2D gel of CcI3 supernatant from 4-day-old cultures (four 100-ml flasks combined). Approximately 150 μg protein was loaded, and the gel was stained with Coomassie blue. (B) 2D gel of CcI3 cytoplasmic extract from 4-day-old cells. Approximately 50 μg protein was loaded, and the gel was silver stained. Protein spots present in both extracts are circled. Three protein spots (boxes) were identified by MS/MS, as follows: spot 1, stress protein (gi 86738980); spot 2, stress protein (gi 86742358); spot 3, phosphoesterase (gi 86739452).

LC MS/MS identification of CcI3 supernatant proteins. A more sensitive way to identify proteins in a complex sample is by using LC MS/MS of tryptic peptides. Silver staining on gels has a visualization limit of about 10 pg/mm^2 (24); LC MS/MS can provide information on individual peptides at 1 to 10 pg. Direct LC MS/MS analysis of proteins from supernatants of 2- to 5-day-old CcI3 cultures yielded 37 protein identifications (Table 1; also see Table SM1 in the supplemental material). Fifteen homologs were detected in supernatants of strain CpI1 in this study, and 10 were detected in ACN14a in a previous study (1).

TABLE 1. Proteins identified by LC MS/MS in the CcI3 culture supernatant[c]

Proteins	gi no.	Annotation	Molecular size (kDa)	No. of replicates	Detection in supernatant:	
					CpI1[a]	ACN14a[b]
Sec signal peptides[d]	86739057	Extracellular ligand-binding receptor (LivK domain)	39.8	1	−	+
	86742940	Periplasmic phosphate binding protein	37.6	2	−	−
Central metabolism proteins	86740018	Aconitate hydratase 1	98.8	3	−	−
	86743039	Fructose-bisphosphate aldolase, class II	36.9	3	−	+
	86738768	Glucose-6-phosphate isomerase	60.4	3	−	−
	86740342	Glyceraldehyde-3-phosphate dehydrogenase, type I	35.6	3	+	−
	86742701	Glyceraldehyde-3-phosphate dehydrogenase, type I	36.2	4	−	−
	86740901	Isocitrate dehydrogenase, NADP-dependent	79.0	2	+	−
	86742576	Phosphoenolpyruvate carboxykinase (GTP)	66.9	3	−	−
	86740343	Phosphoglycerate kinase	40.9	3	+	+
	86742602	Phosphopyruvate hydratase (enolase)	44.8	3	+	+
	86739365	Succinyl-CoA synthetase, α subunit	29.8	1	−	−
	86739364	Succinyl-CoA synthetase, β subunit	40.2	3	−	−
	86740353	Transaldolase	39.8	2	+	−
	86739985	Transketolase-like	65.9	2	−	−
Other metabolism proteins	86742537	4Fe-4S ferredoxin, iron-sulfur binding	11.6	3	+	−
	86742165	Acyl carrier protein	8.5	2	+	−
	86743129	Adenosylhomocysteinase	51.7	3	−	−
	86741632	Alcohol dehydrogenase, zinc-binding	35.1	3	−	−
	86742312	Branched-chain amino acid aminotransferase	34.0	2	−	+
	86740866	Butyryl-CoA dehydrogenase	44.0	2	−	−
	86741123	Cysteine synthase A	32.5	1	−	−
	86741828	Glutamine synthetase, type I	53.8	3	+	−
	86738805	Phosphoserine aminotransferase, putative	39.9	2	+	−
Stress proteins	86743073	Chaperonin GroEL	56.7	2	−	−
	86738980	Stress protein/tellurium resistance protein	20.7	4	+	+
	86742358	Stress protein/tellurium resistance protein TerE	20.2	4	+	+
	86741504	Superoxide dismutase	22.0	3	+	+
Other proteins	86740042	Chlorite dismutase	26.5	1	−	−
	86741905	Glutamyl-tRNA synthetase	51.5	1	−	−
	86738903	Glyoxylase/bleomycin dioxygenase domain	29.6	3	+	−
	86741081	Ham1-like protein	19.2	2	−	−
	86740212	Hypothetical protein Francci3_1507 (DsbA domain)	22.3	2	+	+
	86742100	Hypothetical protein Francci3_3417	11.1	2	−	−
	86742609	Nucleotidyl transferase	31.5	2	−	−
	86739868	Peptidase M1, aminopeptidase N actinomycete-type	95.8	3	+	−
	86739165	Protein of unknown function DUF1416	10.2	1	−	+

[a] LC MS/MS analysis of the *Frankia* sp. strain CpI1 supernatant (this study).
[b] *Frankia alni* strain ACN14a proteins identified from spots on 2D gels of the supernatant (1).
[c] All proteins were identified by Mascot by two or more peptides with ion scores of >15, using the CcI3 genome as a database. CoA, coenzyme A.
[d] Predicted by Signal P v.3.0 (3) to have signal peptides.

Consistent with the overlap observed between the supernatant and cell extract patterns on 2D gels, mainly cytoplasmic proteins were identified by LC MS/MS. With the exception of two proteins predicted to be secreted by the Sec pathway (Table 1), all others are predicted to be cytoplasmic using the LocateP program, a subcellular location predictor (39). Most of the proteins were either small (<30 kDa) or naturally abundant in the cytoplasm. Thirteen of the 37 proteins are enzymes in carbon central metabolic pathways, and others, such as glutamine synthetase and GroEL, are also abundant. Since cells were cultivated on pyruvate, which enters central metabolism through the TCA cycle and is converted to glucose by gluconeogenesis, enzymes of these pathways are highly expressed during growth and are expected to be the first detected during cell lysis.

Some studies have reported that certain glycolytic enzymes are associated with the cell surface in gram-positive bacteria (27). A surface-localized glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in streptococci binds to host extracellular matrix glycoproteins and cytoskeletal proteins, and a GAPDH abundant in the cell wall proteome of *Lactobacillus plantarum* is implicated in adhesion to mucin (17, 28). CcI3 also has a second GAPDH that is homologous to that located on the surface in other gram-positive bacteria. This cognate GADPH gene (gi 86742701) is between hypothetical protein genes, while the major GADPH is among genes encoding other glycolytic enzymes.

Extracellular localization of some other proteins is logical, such as the hypothetical protein (gi 86740212) with a "DsbA" (22.2 kDa; periplasmic disulfide bond oxidoreductase) domain, found in the supernatant of the three strains. Lysyl aminopeptidase activity was measured from CcI3 supernatant extracts,

which is ascribed to "peptidase M1/aminopeptidase N" (gi 86739868), sharing 56% sequence identity with PepN purified from the supernatant of a *Streptomyces* culture (16). PepN may be responsible for cell wall turnover or amino acid scavenging outside the cell, though it does not appear to be exported by the Sec pathway.

Surprisingly, only two proteins detected contained predicted signal peptides for secretion via the Sec pathway: an extracellular ligand-binding receptor with a "LivK" (leucine/isoleucine/valine) conserved domain and a phosphate-binding protein (Table 1). These are not restricted to the supernatant, but were also detected in proteomics analyses of root nodules and cells (see Table 2). The signal peptide-containing phosphoesterase (Fig. 1, spot 3) was not identified by direct LC MS/MS analysis of the supernatant. As only one peptide was identified from the spot excised from the gel (see Table SM1 in the supplemental material), it is possible that this protein is less readily detected by LC MS/MS due to poor trypsin digestion, suboptimal ionization or fragmentation, or masking of peptides by other abundant spectra.

Studies of culture filtrates from the actinobacterium *Mycobacterium tuberculosis* have shown a high proportion of proteins derived from cell lysis, with the exception of a specially prepared filtrate analyzed by Malen et al. (20, 23, 34). Tullius et al. reported that abundantly produced proteins accumulate in the culture supernatant of *Mycobacterium* (35). Other actinobacteria have been reported to secrete substantially more proteins. Roughly 50% of the extracellular proteins of *Corynebacterium diphtheriae* had signal peptides, and 75% of supernatant proteins had signal peptides in *Streptomyces lividans* (10, 14). The low degree of protein secretion in *Frankia* could be related to the medium used. In minimal medium, exported proteins may not be produced; for example, *Bacillus subtilis* and *Bacillus licheniformis* secrete large amounts of protein when grown in rich medium (36). Conversely, in *Streptomyces* species grown in minimal medium, the lack of complex nutrients stimulates cells to secrete proteins in order to scavenge resources (10). Bioinformatics analysis of the three *Frankia* genomes predicted few degradative enzymes, so it is unlikely that the medium alone accounts for the minimal secretome in strain CcI3 (22).

LC MS/MS identification of signal peptide-containing proteins in root nodules. While we can conclude that few proteins are actively secreted in culture under the conditions used, proteins produced in symbiosis that also have predicted signal sequences reveal more about how frankiae function in symbiosis. To study these proteins, we used fractionation techniques to detect *Frankia*-specific proteins among a host of other proteins present in root nodules from three different nodule types. Since the *Frankia* genomes were searched by Mascot to identify peptides, *Frankia* proteins are specifically identified, despite the presence of plant proteins in the nodule extract.

The three sequenced *Frankia* genomes have genes for components of the Sec (general secretory) and Tat (twin arginine translocation) protein secretion pathways (22). At least four of the 10 Sec machinery components were found in each nodule sampled. SecA, YajC, FfH, and FtsY were detected in *Casuarina* nodules; SecA, SecD, and SecF in both *A. incana* and *E. angustifolia* nodules; FtsY in *A. incana*; and SecY in *E. angustifolia*. Since sampling for proteomics is rarely complete, we conclude that the major secretory machinery is synthesized and operational in symbiosis.

Unlike the situation in culture, 42 CcI3 proteins with predicted Sec signal peptides were detected from symbiotic CcI3 in *Casuarina* nodules, out of a total of 1,031 proteins identified by LC MS/MS (our unpublished data). To identify *Frankia* proteins expressed by uncharacterized *Frankia* strains from field-collected root nodules, peptides were matched to sequences from one or more sequenced *Frankia* genomes. Of 1,300 proteins from *A. incana* nodules, 32 with predicted signal peptides were identified when matched to the ACN14a genome; a total of 73 were identified when matched to all three *Frankia* genomes. Similarly, of 1,100 EAN1pec hits in field-grown *E. angustifolia* nodules, 31 with predicted signal peptides were identified when matched to the EAN1pec genome, and 53 were identified when matched with all three genomes.

Table 2 lists the *Frankia* proteins with predicted signal peptides (and two or fewer predicted transmembrane domains) identified from root nodule extracts. The complete list of signal peptide-containing proteins detected (including hypothetical proteins) and the peptide sequences matching each protein are provided in Tables SM2 to SM5 of the supplemental material. LocateP subcellular location predictions are shown to provide some distinction between extracellular and membrane-anchored proteins (39).

The "cell wall/growth" category contains known cell division and cell wall-modifying enzymes, many of which are predicted by LocateP to be released from the cell. Proteins with "OmpA" and "lipopolysaccharide biosynthesis" domains suggest cell envelope modification functions in *Frankia*.

Only one putative secreted hydrolase, a hypothetical protein possessing a metalloprotease domain, was detected in *Casuarina* root nodules, while two other proteases were detected in *E. angustifolia* nodules. Two putative secreted lipases were found in *A. incana* nodules. Surprisingly, the "exported lipase" (gi 111219534) in *E. angustifolia* nodules matched 15 peptides from the ACN14a protein sequence (see Table SM5 in the supplemental material), but did not match any peptides in the genome of the more closely related *Elaeagnus* strain EAN1pec, despite the 59% amino acid identity shared between homologous ACN14a and EAN1pec sequences.

Solute-binding proteins were the most common class of secreted proteins observed in the symbiotic proteomes. All but one were predicted by LocateP to be anchored to the membrane rather than released, which is a typical arrangement for such proteins in gram-positive bacteria (33). Conserved protein domains and top-scoring BLAST hits suggest which nutrients, metabolites, and ions are exchanged between the symbiont and host by the use of ABC transporters. Inorganic ion transport components include molybdate, phosphate, sulfonate, and possibly iron and other metal ions ("TroA" domain). Of the organic substrates, amino acid- and peptide-binding proteins were frequently detected, with the latter found only in the *A. incana* and *E. angustifolia* nodules. Three solute-binding proteins were expressed by CcI3 in *Casuarina* root nodules, but not detected in the proteome of CcI3 in culture. One of these is a "LivK" domain protein (gi 86741514) possessing sequence similarity to *Azorhizobium* and *Bradyrhizobium* proteins (E value of 10^{-44}, with 30% amino acid identity). Proteomic studies have detected expression of branched-chain amino acid

TABLE 2. Signal peptide-containing *Frankia* proteins detected in actinorhizal root nodules[b]

Proteins	*Frankia* gi no.	Annotation	Detection in:				LocateP prediction	LocateP subcellular localization
			Casuarina nodule	*Alnus* nodule	*Elaeagnus* nodule	CcI3 cytoplasm[a]		
Cell wall/growth enzymes	86742989	ATP-dependent metalloprotease FtsH	+	+	+	+	Membrane	Multitransmembrane
	86742986	D-Alanyl-D-alanine carboxypeptidase	−	+	+	−	Extracellular	Secretory (released)
	86742615	Lipopolysaccharide biosynthesis	+	+	−	+	Membrane	Multitransmembrane
	158317879	NAD-dependent epimerase/dehydratase	−	−	+	−	Membrane	N-terminally anchored
	86740618	OmpA/MotB domain protein	+	−	+	+	Extracellular	Secretory (released)
	86738783	Poly-gamma-glutamate biosynthesis protein	−	−	+	−	Membrane	N-terminally anchored
	86742138	Putative lipoprotein	+	−	+	+	Extracellular	Lipid anchored
	86742932	Putative lipoprotein	+	+	−	+	Membrane	N-terminally anchored
	86739919	Rod shape-determining protein MreC	+	+	+	−	Membrane	N-terminally anchored
	86743040	Transglycosylase-like	+	−	−	−	Extracellular	Secretory (released)
	158316857	UDP-*N*-acetylmuramoylalanine-D-glutamate ligase	−	+	−	−	Extracellular	Secretory (released)
Hydrolytic enzymes	86741105	Metalloprotease-like protein	+	+	−	−	Extracellular	Lipid anchored
	158312797	Peptidase S16 Lon domain protein	−	−	+	−	Membrane	N-terminally anchored
	86743135	Polyhydroxybutyrate depolymerase	−	+	+	−	Extracellular	Lipid anchored
	111224419	Putative alkaline serine protease	−	−	+	−	Extracellular	Secretory (released)
	111219534	Putative exported lipase	−	+	+	−	Extracellular	Lipid anchored
	111223109	Putative lipase	−	+	−	−	Extracellular	Secretory (released)
Solute-binding proteins (amino acids)	86740324	Branched-chain amino acid ABC transporter, substrate-binding	−	−	+	−	Extracellular	Secretory (released)
	86739057	Extracellular ligand-binding receptor (LivK domain)	+	+	−	+	Extracellular	Lipid anchored
	86741514	Extracellular ligand-binding receptor (LivK domain)	+	−	−	−	Extracellular	Lipid anchored
	111222939	Putative branched-chain amino acid transport, substrate-binding	−	+	−	−	Extracellular	Lipid anchored
	111222806	Putative branched-chain amino acid transport, substrate-binding	−	+	−	−	Extracellular	Lipid anchored
	86739565	Putative Leu/Ile/Val/Thr-binding protein precursor	+	+	+	+	Extracellular	Lipid anchored
	86742202	Putative glutamate binding protein of ABC transporter system	+	+	−	+	Extracellular	Lipid anchored
	86741119	Extracellular solute-binding protein, family 3 (Gln-binding)	−	+	−	−	Extracellular	Lipid anchored
Solute-binding proteins (peptides)	111222240	Dipeptide-binding lipoprotein	−	+	−	−	Extracellular	Lipid anchored
	158313902	Extracellular solute-binding protein family 5	−	+	−	−	Extracellular	Lipid anchored
	158314797	Extracellular solute-binding protein family 5	−	+	+	−	Extracellular	Lipid anchored
	158318094	Extracellular solute-binding protein family 5	−	+	+	−	Extracellular	Lipid anchored
	158315708	Extracellular solute-binding protein family 5	−	−	+	−	Extracellular	Lipid anchored
	158316615	Extracellular solute-binding protein family 5	−	+	−	−	Extracellular	Lipid anchored
	158317996	Extracellular solute-binding protein family 5	−	+	−	−	Extracellular	Lipid anchored
Solute-binding proteins (inorganic ions)	86742252	ABC transporter, aliphatic sulfonate-binding	+	+	+	+	Extracellular	Lipid anchored
	158317681	Periplasmic binding protein (FepB domain, iron-binding)	−	−	+	−	Extracellular	Lipid anchored
	158314107	Periplasmic molybdate-binding protein	−	+	+	+	Extracellular	Lipid anchored
	86742940	Periplasmic phosphate-binding protein	+	+	−	+	Extracellular	Lipid anchored
	86741175	Periplasmic solute-binding protein (TroA domain)	+	−	−	+	Extracellular	Lipid anchored
	86738968	Putative sulfonate-binding protein precursor	+	−	−	−	Extracellular	Lipid anchored
Solute-binding proteins (other)	86741521	Extracellular solute-binding protein family 1	+	−	−	−	Extracellular	Lipid anchored
	158318778	Extracellular solute-binding protein family 1	−	−	+	−	Extracellular	Lipid anchored
	158317895	Extracellular solute-binding protein family 1	−	+	+	+	Extracellular	Lipid anchored
	111221811	Putative ABC transporter substrate-binding protein	−	+	−	−	Extracellular	Lipid anchored
	111223273	Putative ABC transporter, membrane-binding protein	−	+	−	−	Extracellular	Lipid anchored
Proteins involved in cell processes	86742499	Alkaline phosphatase	−	−	+	−	Membrane	N-terminally anchored
	86739476	Amidophosphoribosyltransferase-like	+	−	−	−	Membrane	N-terminally anchored
	86742209	DEAD/DEAH box helicase-like	+	+	−	+	Membrane	N-terminally anchored
	86739994	Diguanylate cyclase/phosphodiesterase	+	+	+	−	Membrane	Multi-transmembrane
	158315891	D-Isomer-specific 2-hydroxyacid dehydrogenase NAD-binding	−	+	−	−	Cell wall	LPxTG cell wall anchored
	86740326	DNA polymerase I	+	+	+	+	Extracellular	Secretory (released)
	86742982	DNA-directed DNA polymerase	+	−	−	−	Membrane	N-terminally anchored
	86740098	FAD dependent oxidoreductase	−	+	+	−	Extracellular	Secretory (released)
	86738950	FMN-binding	−	+	−	−	Extracellular	Secretory (released)
	86739182	Glycine cleavage T protein aminomethyl transferase	−	+	−	+	Membrane	N-terminally anchored
	158311946	Integral membrane sensor signal transduction histidine kinase	−	−	+	−	Membrane	Multitransmembrane

Continued on following page

TABLE 2—*Continued*

Proteins	*Frankia* gi no.	Annotation	Detection in:				LocateP prediction	LocateP subcellular localization
			Casuarina nodule	*Alnus* nodule	*Elaeagnus* nodule	CcI3 cytoplasm[a]		
	86742607	MazG family protein	−	+	−	−	Membrane	N-terminally anchored
	86741365	N-6 DNA methylase	+	+	−	−	Extracellular	Secretory (released)
	86739036	Periplasmic sensor signal transduction histidine kinase	−	+	+	−	Membrane	Multitransmembrane
	86741547	Periplasmic sensor signal transduction histidine kinase	−	+	−	+	Membrane	Multitransmembrane
	86740996	Protoporphyrin IX magnesium-chelatase	−	+	+	−	Extracellular	Secretory (released)
	111223706	Putative copper resistance protein partial match	−	+	−	−	Membrane	C-terminally anchored
	111223594	Putative hybrid sensor histidine kinase/response regulator	−	+	−	+	Membrane	Multitransmembrane
	111223825	Putative IS*605* family transposase	−	−	+	+	Extracellular	Secretory (released)
	111222332	Putative modular polyketide synthase	−	+	+	+	Membrane	N-terminally anchored
	111223507	Putative non-ribosomal peptide synthetase; putative signal peptide	−	+	+	−	Membrane	N-terminally anchored
	111221481	Putative *S*-adenosyl-L-methionine-dependent methyltransferases	−	+	−	−	Membrane	N-terminally anchored
	158312253	Putative sensor with HAMP domain	−	+	−	+	Membrane	Multitransmembrane
	111219913	Putative short-chain dehydrogenase/reductase	−	+	−	+	Membrane	N-terminally anchored
	158313514	Secreted protein	−	−	+	−	Extracellular	Secretory (released)
	158317049	Stage II sporulation E family protein	−	−	+	−	Membrane	Multitransmembrane
	86741563	Superoxide dismutase, copper/zinc binding	+	−	−	+	Membrane	N-terminally anchored
	86739099	Thiamine biosynthesis protein ThiC	+	−	+	+	Membrane	N-terminally anchored
	111225298	Threonine synthase	−	+	−	+	Extracellular	Secretory (released)
	86742403	Transcription termination factor Rho	+	+	+	+	Membrane	N-terminally anchored
	86740221	Transcriptional regulator, MerR family	−	+	−	−	Extracellular	Secretory (released)
	86740783	Transcriptional regulator, TetR family	+	+	−	−	Extracellular	Secretory (released)
	158312181	Type II secretion system protein	−	−	+	+	Membrane	Multitransmembrane
	86741605	von Willebrand factor, type A	−	−	+	+	Extracellular	Lipid anchored
	86740906	YceI	+	−	−	+	Membrane	N-terminally anchored

[a] Data from multidimensional LC MS/MS analysis of cytoplasmic extract from cultured CcI3 cells provided as a comparison to CcI3 in *Casuarina* nodules (our unpublished data).

[b] All proteins were predicted to contain signal peptides by both methods of SignalP v. 3.0 (Neural Networks and Hidden Markov Models), and predicted to contain zero to two transmembrane domains by TMHMM v. 2.0. Proteins were identified by Mascot by two or more significant peptides with ion scores of >15, using the CcI3 genome as a database for *Casuarina* nodule proteins and CcI3 cytoplasm, and either the CcI3, ACN14a, or EAN1pec genome for the *Alnus* and *Elaeagnus* nodule proteins.

transporters in *Bradyrhizobium* and *Sinorhizobium* symbiotic bacteroids (9, 31). It is noteworthy that branched-chain amino acid-binding and peptide-binding protein genes fall into the top 20 duplicated gene families in both the ACN14a and EAN1pec genomes (26). Thus, amino acid transport in symbiosis may be an important component of bacterium-plant interactions.

An intriguing finding was the presence of multiple "extracellular solute-binding family 5" proteins, predicted to bind oligopeptides, in field-collected nodules. This provides another instance of an environmental *Frankia* strain, in this case the *A. incana* symbiont, expressing a protein more similar to a heterologous frankial sequence (EAN1pec). The EAN1pec genome contains seven paralogous "family 5" proteins, but the CcI3 and ACN14a genomes lack clear orthologs. The top-scoring BLAST hits to these duplicated EAN1pec sequences are putative peptide-binding proteins in *Agrobacterium tumefaciens* (31% identity, E value of 10^{-60}) and *Rhizobium leguminosarum* (30% identity, E value of 10^{-55}). Other proteins involved in a variety of cell processes, including transcriptional regulation, have signal peptides predicted. Many are associated with the membrane and are thus not secreted outside the cell.

Conclusions. The gradual appearance of protein spots on 2D gels of supernatants as cultures age, the identity of peptides in the supernatant determined by LC MS/MS, and the observation that most members of the exoproteome were abundant and/or of small molecular size suggest cell lysis during growth rather than targeted secretion. It is probable that additional secreted proteins in *Frankia* remain either anchored to the membrane or associated with the cell envelope and are thus not found in the medium.

Symbiotic *Frankia* cells, prepared by sonication, yielded a greater number of cell wall-bound or membrane-bound proteins with signal peptides. In *Casuarina* root nodules and in field-collected nodules of other actinorhizal species, solute-binding proteins emerged as the most common class of secreted proteins. In gram-positive bacteria, solute-binding proteins are generally tethered to the cell wall, in contrast to gram-negative bacteria, where most reside in the periplasm (33). Branched-chain amino acid-binding proteins were detected in all nodules, but biochemical characterization is necessary to determine which ligands are bound.

Perhaps the most striking aspect of this study is that hydrolytic enzymes were rarely detected in either culture or symbiosis, indicating that plant cell wall or membrane digestion is not likely to be used by *Frankia* cells colonizing plant cells inside root nodules. Most plant pathogens secrete cellulases, pectinases, xylanases, or other enzymes to hydrolyze plant cell wall polymers (18, 29, 37). The lack of secreted hydrolases has been proposed to be favorable for microorganisms that form beneficial associations with plants (21, 25). Evidence presented here and reported in a previous bioinformatics study (22) that

showed a diminutive secreted proteome strongly suggests that frankiae fall into this schema.

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