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Bacteria Associated with Cysts of the Soybean Cyst Nematode (*Heterodera glycines*)

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The soybean cyst nematode (SCN), *Heterodera glycines*, causes economically significant damage to soybeans (*Glycine max*) in many parts of the world. The cysts of this nematode can remain quiescent in soils for many years as a reservoir of infection for future crops. To investigate bacterial communities associated with SCN cysts, cysts were obtained from eight SCN-infested farms in southern Ontario, Canada, and analyzed by culture-dependent and -independent means. Confocal laser scanning microscopy observations of cyst contents revealed a microbial flora located on the cyst exterior, within a polymer plug region and within the cyst. Microscopic counts using 5-(4,6-dichlorotriazine-2-yl)aminofluorescein staining and in situ hybridization (EUB 338) indicated that the cysts contained (2.6 ± 0.5) × 10^5 bacteria (mean ± standard deviation) with various cellular morphologies. Filamentous fungi were also observed. Live-dead staining indicated that the majority of cyst bacteria were viable. The probe Nile red also bound to the interior polymer, indicating that it is lipid rich in nature. Bacterial community profiles determined by denaturing gradient gel electrophoresis analysis were simple in composition. Bands shared by all eight samples included the actinobacterium genera *Actinomadura* and *Streptomyces*. A collection of 290 bacteria were obtained by plating macerated surface-sterilized cysts onto nutrient broth yeast extract agar or on actinomycete medium. These were clustered into groups of siblings by repetitive extragenic palindromic PCR fingerprinting, and representative isolates were tentatively identified on the basis of 16S rRNA gene sequence. Thirty phylotypes were detected, with the collection dominated by *Lysobacter* and *Variovorax* spp. This study has revealed the cysts of this important plant pathogen to be rich in a variety of bacteria, some of which could presumably play a role in the ecology of SCN or have potential as biocontrol agents.

The soybean cyst nematode (SCN), *Heterodera glycines*, is an important pathogen of soybeans (*Glycine max*) worldwide (1). This nematode has a broad host range including other legumes, some ornamentals, and a number of common weeds. Although it is an obligate plant pathogen, important components of its life cycle take place in the soil outside the plant host. At the end of their lives, females that have been feeding on soybean roots become engorged with eggs, encyst, die, and are shed from the roots into the surrounding soil. Encysted eggs can remain viable for many years as a reservoir of infection for subsequent soybean crops (1). Although there are SCN-resistant soybean cultivars, control of this pest currently consists largely of rotating to nonhost crops, allowing nematode infestations to decline to levels which result in economically tolerable yield loss of subsequent soybean crops.

There is some evidence that SCN populations in soil are suppressed by both fungal and bacterial parasites. Both encysted eggs and the active infective juvenile nematodes are subject to fungal parasitism (5, 8). Some fungi are potential biological control agents (8, 49). The obligate nematode endoparasitic bacterium *Pasteuria* sp. may suppress SCN populations in some soils and likewise has potential for biocontrol of a variety of plant-parasitic nematodes (3, 10, 49). Several bacteria have been found to inhibit infection of crops by cyst nematodes, either through direct inhibition or parasitism of the nematode or through host plant-induced resistance (13, 18, 20, 45, 53).

Overall, these results indicate that a better understanding of the interactions of microorganisms and SCN in soil should yield new insights into the ecology of this pathogen and perhaps new biocontrol approaches (4). The aim of the study reported here was to explore the diversity and identity of bacteria associated with cysts of SCN.

MATERIALS AND METHODS

Handling of soils and SCN cysts. Soils from eight SCN-infested farms were sampled in southern Ontario, Canada (within a 100-km radius of 42′60″N 83′W). The soils varied widely in texture (from sandy loam containing 74% sand and 7% clay to clay loam containing 30% clay and 34% sand), pH (5.2 to 7.5), and organic matter content (1.6 to 6.6%).

Bulk soil samples (100 to 500 g depending on level of SCN infestation) from each farm were fractionated with flowing water through 0.84-mm- and 212-μm-pore-size stacked sieves. The particles recovered on the finer mesh were rinsed into a beaker and transferred under gentle vacuum onto filter paper. Using a low-magnification stereoscope, 75 cysts were manually collected from each sample, and these were stored for up to 24 h in distilled H_2O at 4°C.

Cysts were surface sterilized by immersion for 5 min in 0.3% hypochlorous bleach, followed by seven rinses in sterile distilled water. In preliminary experiments to establish the minimum bleach contact time for surface sterilization, cysts were dipped in a cell suspension of *Escherichia coli*, placed on eosin-methylene blue agar following various times of immersion in bleach, and incubated overnight at 30°C. Immersion of cysts in 0.3% hypochlorous bleach for 5

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TABLE 1. Oligonucleotide probes, target organisms, and stringencies used for hybridization

Probe	Target organisms	FA[a] (%)	NaCl concn (mM)	Reference
EUB 338	*Bacteria*	20	250	2
ALF1b	α-*Proteobacteria*	20	250	35
BET42a	β-*Proteobacteria*	35	88	35
GAM42a	γ-*Proteobacteria*	35	88	35
CF319a/b	*Cytophaga-Flavobacterium* cluster	20	250	36
LGC354c	Gram-positive bacteria with low GC content of DNA	20	250	37
HGC69a	Gram-positive bacteria with high GC content of DNA	35	88	46
SRB385Db	Most members of δ-*Proteobacteria*, including *Desulfobacteriaceae*	35	88	43
ARCH915	*Archaea*	20	250	55

[a] FA, formamide in hybridization buffer.

min, followed by seven rinses in sterile distilled water, was found to be the minimum time to eliminate detectable microbial growth on eosin-methylene blue. Furthermore, this treatment presumably damaged bacterial DNA to the point where it was not amplifiable by PCR, suggesting that denaturing gradient gel electrophoresis (DGGE)-PCR analysis of cyst DNA would not detect surface-associated bacteria. This is based on the observation that suspensions of *E. coli* incubated for 5 min in 0.3% bleach, then recovered by centrifugation and resuspension in sterile distilled water seven times, were not amplifiable using universal 16S rRNA gene primers. Bleach-treated *E. coli* cells did not inhibit amplification of untreated *E. coli* cells when mixed 1:1, indicating that there was no carryover of a PCR inhibitory substance from the sterilization treatment.

Surface-sterilized cysts were placed into a Bio 101 DNA extraction kit and mechanically broken with a FastPrep instrument (three rounds of 30 s at a setting of 5.5; Savant, Holbrook, N.Y.) to release DNA. DNA was extracted from the lysate with the Ultra Clean soil DNA kit (MOBIO Laboratories, Inc., Solana Beach, Calif.) as described by the manufacturer. DNA was extracted from 0.25-g portions of soil using the same procedures as for the cysts.

DNA methods. DNA extracted from surface-sterilized cysts was amplified for DGGE analysis using the universal 16S rRNA gene eubacterial primers GC-GM5F (bp 341 to 357 in the 16S rRNA gene of *E. coli*) and 907R (bp 907 to 928 in the 16S rRNA gene of *E. coli*) (48). Conditions for PCR amplification, DGGE, excision of bands, cloning, sequencing, and analysis of resolved PCR products were exactly as previously described (31).

Preparation of template, composition of PCR mixtures, and thermocycler programming for amplification of DNA using universal eubacterial 16S rRNA gene primers or repetitive extragenic palindromic (REP) fingerprinting using the BOXA1R primer were as previously described (57).

Preparation of cysts for confocal laser scanning microscopy. Cysts were mounted, dissected, and fixed as follows. For microdissection the cysts were mounted in a thin layer of acid-free silicone coating (WPI Inc., Sarasota, Fla.) in a petri plate (9 by 50 mm). They were then opened using microscissors to allow exposure of cyst contents and ready access for staining. Cysts were maintained in a hydrated state throughout the procedures. The cysts used for hybridization had the plug end aseptically excised and then the contents of the cyst were smeared on a slide prior to fixation. Fixation was done essentially following the protocol of Manz et al. (36). Some minor modifications were introduced. The cyst smear was gently heat fixed to the glass slide and then soaked with a formaldehyde solution (3.7%, vol/vol) and fixed for at least 1 h at 7°C. Slides were washed once with 1× phosphate-buffered saline (PBS) (130 mM NaCl, 10 mM Na-phosphate buffer, pH 7.2) for 3 min, air dried, dehydrated in an ethanol series (50, 80, and 96%; 3 min each) and dried at room temperature.

In situ hybridization. Oligonucleotide probes, references, and target organisms used in this study are summarized in Table 1. Oligonucleotides were stored in TE buffer (10 mM Tris, 1 mM EDTA [pH 7.5]) at −20°C. Working solutions were adjusted to 50 ng of DNA per μl. Prewarmed hybridization buffer (0.9 M NaCl, 20 mM Tris-HCl [pH 7.2], 0.01% sodium dodecyl sulfate, formamide concentration as given in Table 1) was mixed with fluorescently labeled oligonucleotide (1 ng/μl of hybridization buffer) and applied to the fixed smear of cyst contents. The slides were placed in humid chambers and incubated for 90 min at 46°C. After this, hybridization buffer was drawn off with tissue placed at the edges of the slides. Subsequently, slides were transferred to 50 ml of prewarmed washing buffer (20 mM Tris-HCl, 0.01% sodium dodecyl sulfate, NaCl concentration as given in Table 1) and incubated at 48°C for 20 min. For microscopic analysis, slides were carefully rinsed with distilled water, air dried, and mounted in antifading glycerol medium (Citifluor AF2; Citifluor Ltd., London, United Kingdom). All hybridization and washing steps were performed in the dark.

Fluorescent stains. A panel of fluorescent probes targeting specific potential cyst components were used to stain the cysts and cyst contents. These included the following lectin conjugants, *Phaseolus vulgaris*-tetramethyl rhodamine isothiocyanate (TRITC), *Wisteria floribunda*-Alexa568, *Tetragonolobus purpureas*-CY5, *Naja naja kaouthia*-Alexa488, *Triticum vulgaris*-CY5, *Solanum tuberosum*-fluorescein isothiocyanate (FITC), *Bandeira simplicifolia*-TRITC, *Lens culinaris*-CY5, and *Ulex europeaus*-CY5. Fluorescent lectins with FITC or TRITC labeling were purchased (Sigma, St. Louis, Mo.). The lectins were employed alone or in combination as described in detail by Neu et al. (41). In addition, the nucleic acid specific stains SYTO9 and SYTO62 were used to stain bacteria and other components of the cysts. The protein stain Sypro Orange and phalloidin-Alexa488 (Molecular Probes Inc., Eugene, Oreg.), which binds to actin, were also applied.

CLSM image collection and analysis. An MRC 1024 confocal scanning laser microscope (CLSM) (Bio-Rad, Hemel Hempstead, United Kingdom), equipped with a krypton-argon laser and mounted on a Microphot SA microscope (Nikon, Tokyo, Japan), was used to obtain images of stained cysts. The cysts were observed using the three excitation-emission lines of the krypton-argon laser (30). Samples were also examined using transmitted laser illumination, autofluorescence, reflection and standard epifluorescence microscopy techniques (30). Observations were made with 60× and 100×, 1.4 numerical aperture (NA) oil immersion lenses, a 20×, 0.75 NA lens, and water-immersible 40×, 0.55 NA (Nikon) and 63×, 0.9 NA (Zeiss, Jena, Germany) lenses.

Image analyses were performed using NIH Image version 1.61 (http://rsb.info.nih.gov/nih-image/) with a macro written for semiautomated quantification as described by Manz et al. (34). CLSM images (20 microscope fields/cyst/probe in duplicate) were collected for image analysis purposes.

Isolation and characterization of bacteria residing in SCN cysts. Twenty-five cysts from each soil were surface sterilized, resuspended in 50 μl of water, macerated in a microcentrifuge tube with a small plastic pestle, and diluted in sterile distilled water. A portion of the macerate was stained with the live-dead staining kit of Molecular Probes and enumerated by epifluorescent microscopy. Serial dilutions of the same samples prepared from soil samples 1, 3, 4, 6, and 7 were enumerated by plate count on 1/10 strength Trypticase soy agar, 1/10 strength nutrient broth yeast extract agar (NBYEA), actinomycete agar, and soil extract agar incubated at 30°C (composition in reference 58). The actinomycete medium had the following composition (in grams/liter): potato starch, 10; casein, 0.3; KNO_3, 2; NaCl, 2; K_2HPO_4, 2; $MgSO_4 \cdot 7H_2O$, 0.05; $CaCl_2$, 0.005; $FeSO_4 \cdot 7H_2O$, 0.005; agar, 15. The pH was adjusted to 7.2 prior to autoclaving, and cycloheximide (50 mg/liter) was added to the cooled agar. All enumeration data are presented as mean ± standard deviation.

The isolates described in this study were all obtained from the actinomycete and NBYEA media. All well-isolated colonies on selected plates were isolated to get a representative sampling, purified on the isolation medium, and frozen in 15% glycerol at −70°C. Siblings were identified by genomic fingerprinting using the BOXA1R primer, and a representative from each fingerprint group was identified on the basis of the sequence of the 16S rRNA gene sequence at positions 341 to 928, directly amplified, sequenced, and analyzed as described above (57). In some cases other regions of the gene were sequenced using previously described primers (29).

Nucleotide sequence accession numbers. Sequences for DGGE bands A to I have been deposited in GenBank and given accession numbers AF543360 to AF54337, and sequences for isolates have been given accession numbers AF547025 to AF547054.

RESULTS

Microscopic observations of cyst contents. Total bacterial populations in cysts sampled from soils 1, 3, and 6 were enumerated by epifluorescence microscopy of live-dead-stained macerated cyst preparations. Soil samples 1, 3, and 6 had $(4.8 \pm 1.2) \times 10^5$, $(4.8 \pm 1.5) \times 10^5$, and $(6.1 \pm 1.3) \times 10^5$ bacteria/cyst, respectively (unless otherwise noted, values are means ± standard deviations; $n = 3$ in each soil). These values were in very close agreement with enumerations done by in situ hybridization with the EUB 338 probe [$(2.6 \pm 0.5) \times 10^5$]. Based on the relative proportion of green- and red-stained

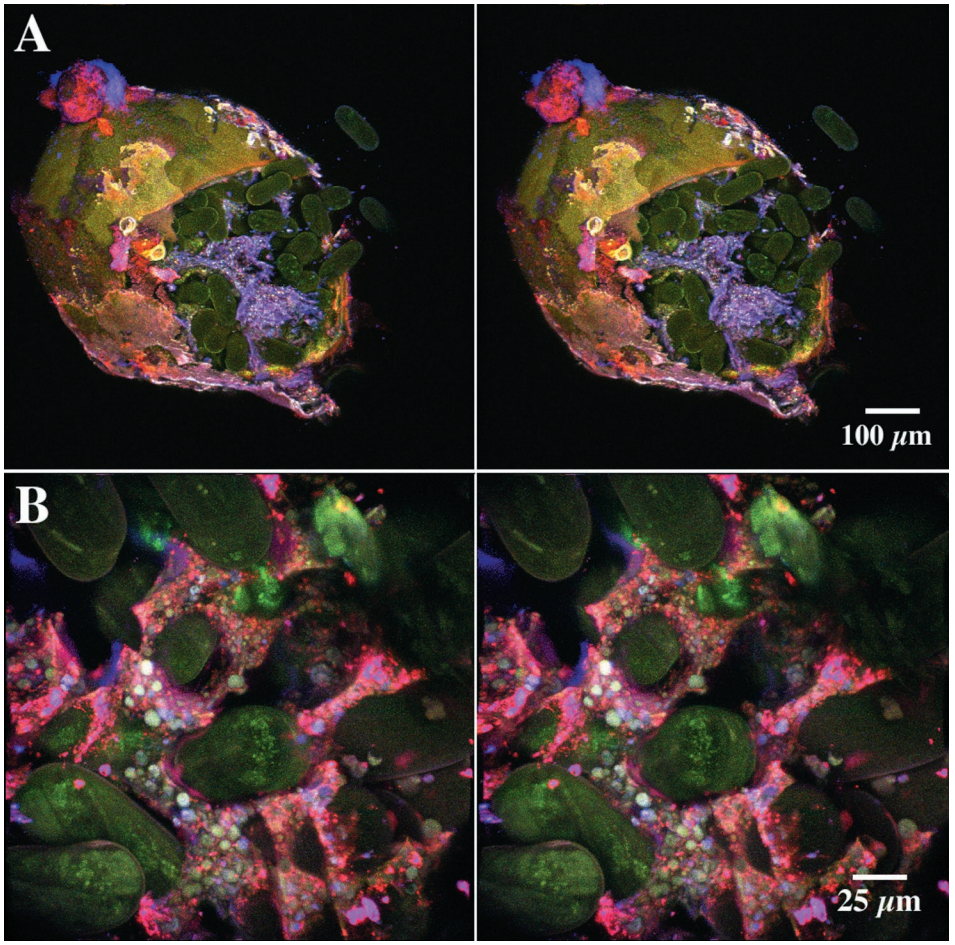

FIG. 1. (A) Low-magnification CLSM stereo projections illustrating the general appearance of the cysts, cyst plug region, cyst wall, and contents including mucilage, eggs and associated bacteria. The cyst has been stained with SYTO62 for bacteria (blue) and *Phaseolus vulgaris*-TRITC (red) and imaged using autofluorescence (green). Eggs appear dark green, whereas the exterior cyst wall which autofluoresces and binds the two probes appears light green. There is a high density of bacteria associated with the "plug region" of the cyst and the exterior cyst wall. (B) High-magnification stereo images of the cyst interior after triple labeling with the lectins *Solanum tuberosum*-FITC (green), *Bandeira simplicifolia*-TRITC (red), and *Lens culinaris*-CY5 (blue); details of the form and nature of the eggs and surrounding mucilage are shown. The mucilage appears heterogeneous in nature, with globular structures and variable lectin staining. Images are best viewed using stereo glasses.

cells in live-dead-stained preparations, 85% ± 4% in cysts from soil 1, 83% ± 6% in cysts from soil 3, and 61% ± 25% in cysts from soil sample 6 were viable.

Cyst morphology is shown in a low-magnification three-color stereo pair (Fig. 1A). The cysts have an elaborate outer coat which binds a broad range of fluorescent lectins and the probes Sypro Orange and phalloidin-Alexa488. The inside of the cyst contains eggs which are embedded in a heterogeneous mucilaginous material which stains with a limited range of probes including Nile red and some lectins (Fig. 1B). The plug layers and outer shell of the cysts were extensively colonized by bacteria and other soil microorganisms (Fig. 1A and Fig. 2B). Figure 2 shows detailed examination of cyst plug, wall, and interior materials using nucleic acid staining of fully hydrated materials. The presence of abundant populations with various morphologies dominated by rod-shaped cells is evident particularly on the surface of the cysts. Bacteria found among the eggs were less numerous and diverse in nature (Fig. 2C). Microscopic observations of dividing cells and cells hybridizing with the EUB 338 probe were consistent with other evidence of an active microbial community associated with and within the cysts. Total populations observed with EUB 338 also agreed with the sum of populations determined with the more specific probes (Fig. 3). In situ analysis using a comprehensive suite of rRNA-targeted, domain- and group-specific probes visualized individual cells within the alpha-, beta-, and gamma-*Proteobacteria* as well as the *Cytophaga-Flavobacterium* group as major parts of the community. Members with affiliation to the low- and high-GC-content gram-positive bacteria and sulfate-reducing bacteria were also detected in the cyst contents. Gamma-*Proteobacteria* constituted the most abundant group within the cyst communities. The presence of sulfate-reducing bacteria indicated a range of metabolic potential within the cysts. Hybridizations using the archaeon 905 probe did not indicate the presence of these bacteria within the community.

PCR-DGGE analysis of SCN cyst eubacterial communities. The 16S rRNA gene-DGGE profiles prepared with DNA isolated from the cysts typically revealed 12 to 24 bands, with 84 discrete bands in total visible (Fig. 4). This is in contrast to DGGE profiles prepared from bulk soil, which yielded unin-

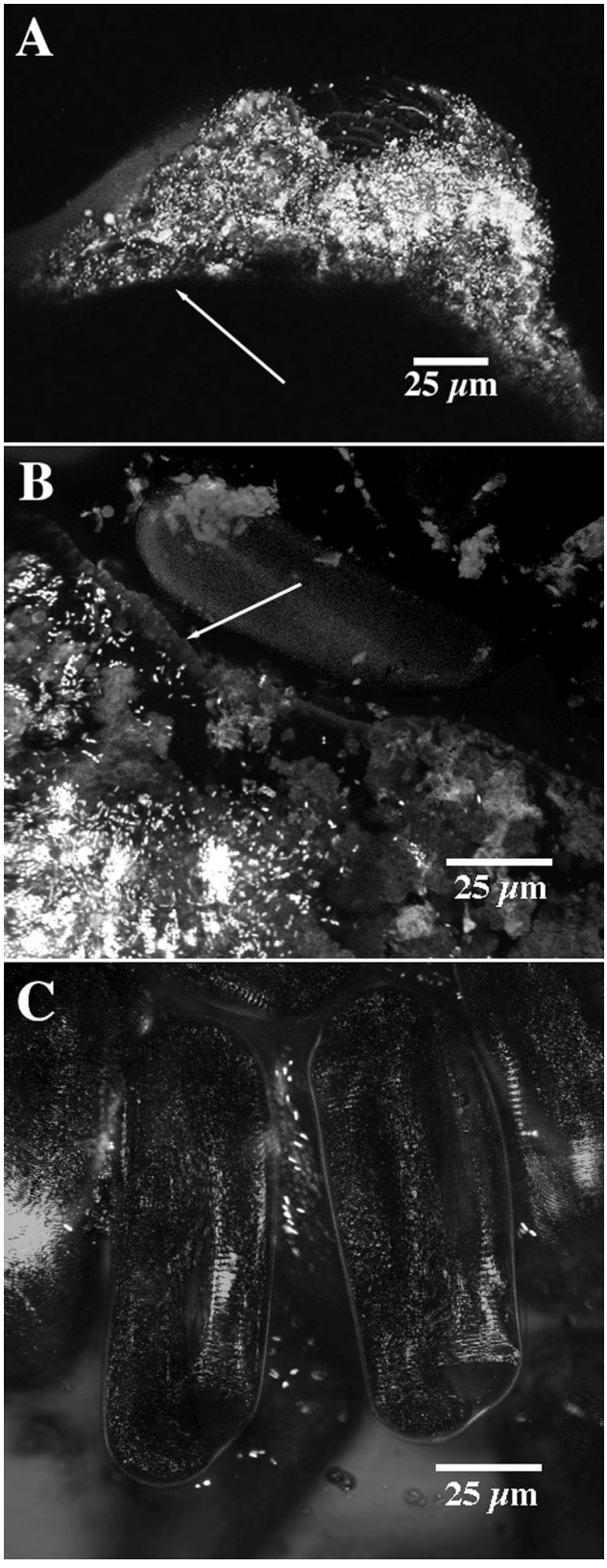

FIG. 2. CLSM images illustrating detail of the cyst plug, wall and interior matrix regions of the cyst material. (A) Detail of plug stained with SYTO9 and imaged using red/blue autofluorescence, at higher magnification than in Fig. 1, illustrating the form and distribution of cells, microcolonies and associated materials in this region of the cyst. (B) Greyscale image (SYTO9) increased magnification of the cyst wall showing bacterial masses on the outside of the cyst. (C) Confocal micrograph of nematode eggs and associated bacteria stained with SYTO9; eggs and developing nematodes were imaged using a combination of reflectance and phase-contrast transmission. Arrows indicate the location of the cyst wall.

terpretable smears (data not shown). Every sample had a distinct community profile with numerous bands unique to individual samples, but some prominent bands (e.g., bands F, G, and I) were found in all cyst samples. Major bands from cyst sample 1 were cloned, sequenced, and tentatively identified on the basis of comparison with sequences available in GenBank (Table 2). Band F was most closely related to a clone prepared from a rhizosphere DNA sample. Band G was most closely related to the genus *Actinomadura*, and band I to the genus *Streptomyces*. Other bands were related to *Flexibacter-Cytophaga-Sphingobacterium-Flavobacterium* (bands A and B), unidentified soil bacterial clones (bands C and D), and the actinomycete genus *Lentzea* (band I).

Identity of bacteria cultured from SCN cysts. Bacterial populations enumerated by plate count on 1/10 strength Trypticase soy agar ranged from 1.2×10^3 CFU/cyst (soil 6) to 1.2×10^4 CFU/cyst (soil 4); those on 1/10 NBYEA ranged from 10^3 CFU/cyst (soil 6) to 1.4×10^4 CFU/cyst (soil 4); those on soil extract agar ranged from 1.5×10^3 CFU/cyst (soil 6) to 1.6×10^4 CFU/cyst (soil 1); and those on actinomycete agar ranged from 10 CFU/cyst (soil 7) to 240 CFU/cyst (soil 1).

The 290 isolates described in this study (Table 3) were all obtained from the enumerations on actinomycete and NBYEA media. In order to get a representative sampling, all of the colonies from plates with well-dispersed colonies were taken. Isolates were purified by repeated streaking on the isolation medium and grouped into siblings on the basis of REP-PCR fingerprinting with the BOXA1R primer. A representative from each fingerprint group was tentatively identified on the basis of the sequence of the 16S rRNA gene (positions 341 to 928), directly amplified, sequenced, and analyzed as described in reference 57 (Table 2). A total of 97 distinct fingerprints were obtained, yielding a total of 30 distinct phylotypes on the basis of the partial sequence of the 16S rRNA gene, in most cases with % similarities to the closest relative in GenBank of ≥97%. Almost a third of the isolates were most closely related to *Variovorax* sp. and a fifth of the isolates were closely related to *Lysobacter antibioticus*, which was also the most widespread phylotype, having been isolated in four of the five soils sampled. Three other phylotypes (*Dyadobacter fermentans*, *Variovorax* sp. strain P9G781, and *Streptomyces neyagawaensis*) were isolated from three soils, five phylotypes (*Variovorax* sp. strain P16G917, *Ultramicrobacterium* sp. strain 12-3, *Microbacterium trichotechnolyticum*, *Rhodococcus* sp. strain RHA7, and *Flexibacter sancti*) were isolated from two soils, and the remainder were isolated from a single soil.

DISCUSSION

This study has revealed that cysts of this important soybean pathogen are rich in bacteria. Cyst samples obtained from a variety of farms consistently contained 10^5 bacteria enumer-

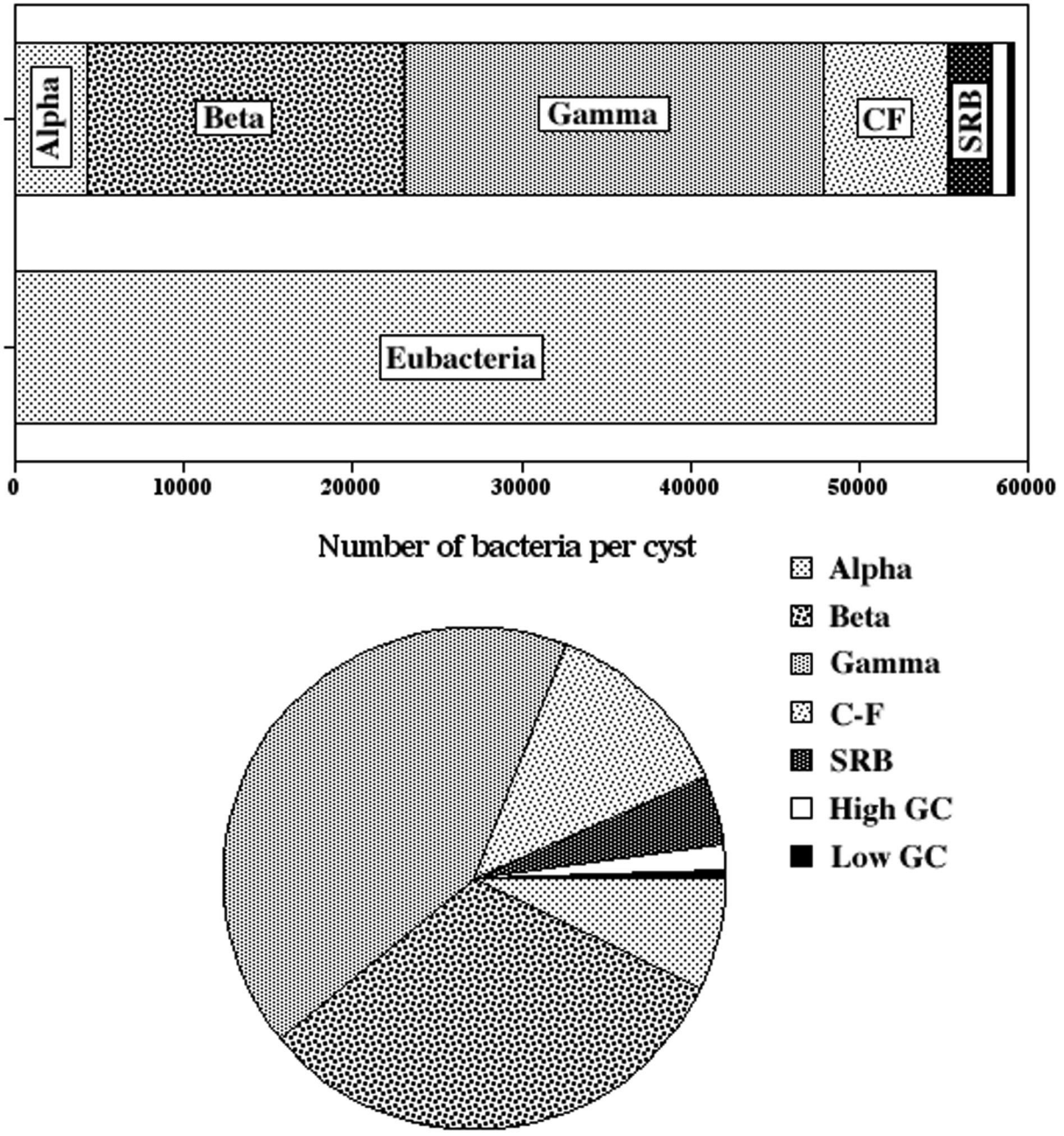

FIG. 3. Graph showing the total cell areas covered by different taxonomic groups of cyst bacteria after hybridization with oligonucleotide probes specific for *Bacteria*, alpha- and beta-, as well as gamma-*Proteobacteria* and the *Cytophaga-Flavobacterium* group. In addition, results of probes for high- and low-GC gram-positive bacteria and for sulfate-reducing bacteria were also quantified.

ated by microscopic observation of preparations treated with fluorescent stains or with the EUB 338 probe. From microscopic observations we estimate the average cyst volume to be about 0.1 μl, and thus the bacterial density within the cysts corresponded to about 1.0×10^9 bacteria/ml of cyst volume. Observation of cyst preparations stained with the live-dead fluorescent probe suggested that the majority of the bacteria were viable. We have not attempted to follow the bacterial complement of cysts over time, but presumably the abundance and composition of the bacterial community will change following the release of a newly formed cyst from an infected root into the soil. Cysts of SCN and other members of the genus *Heterodera* have two openings, the mouth and the vulva, through which hatched larvae can escape; they are thus open to bacteria in the soil. Chemical analyses of cysts of *Globodera rostochiensis* (golden cyst nematode) indicated that mature cysts were rich in lipid materials including triacylglycerols, ethanolamine phosphoglycerides, choline phosphoglycerides, and a total phospholipid content of 12.8% (16). Fatty acids were also present in the cysts. Thus, there is likely to be a range of

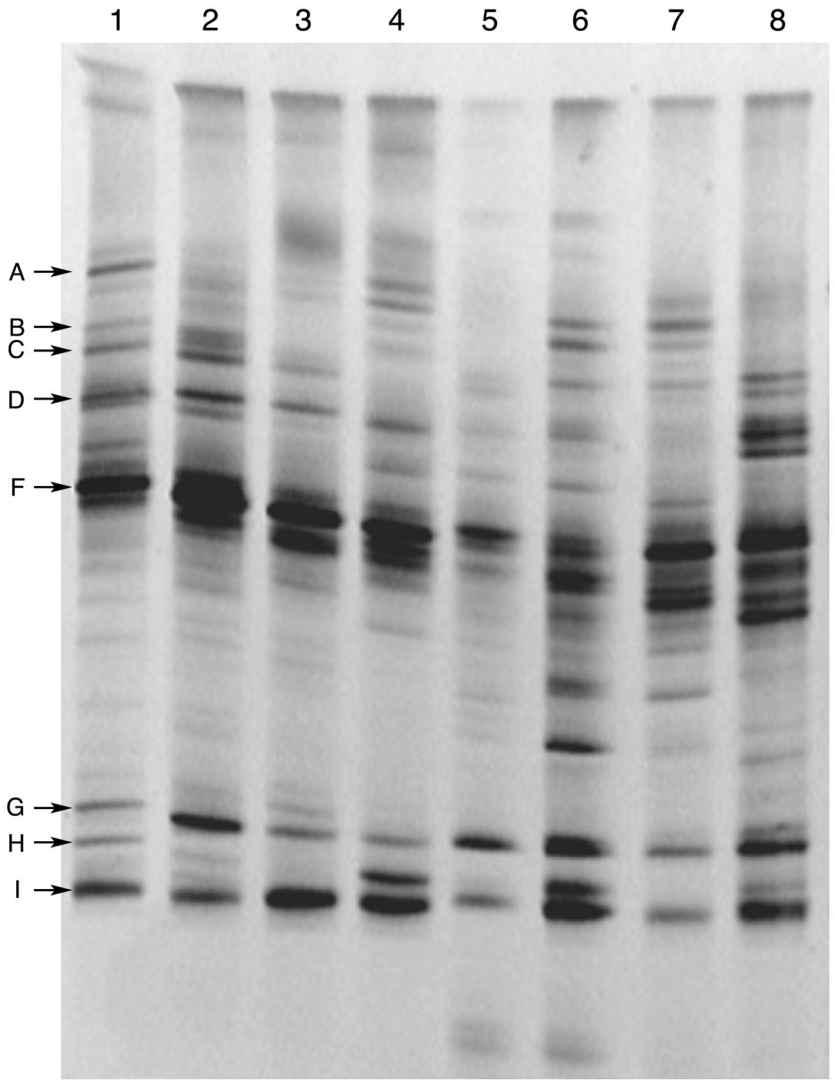

FIG. 4. DGGE analysis of DNA isolated from cysts and amplified with universal eubacterial 16S rRNA gene primers. Each lane represents cysts from a single SCN-infested farm. The indicated bands in sample 1 were cloned and sequenced (Table 2).

available carbon to sustain active microbial populations in the cysts.

The distribution of bacteria in the cyst was heterogeneous. As shown in Fig. 1 and 2, bacteria were associated with both the oral or vulval plug and throughout the cysts' interior mucilage. Based on indices such as live-dead staining, EUB 338 hybridization, and detection of dividing cells, the microbial community was viable and active. Although there were a range

TABLE 2. Identity of DNA bands in DGGE-16S rRNA gene analysis of cyst bacterial communities[a]

Band	Closest relative	% Identity (no. identical/total no.)	Accession no. of closest relative	Reference
A	*Flexibacter elegans*	94 (538/569)	M58782	This study
B	*Sphingobacterium* sp.	98 (570/579)	AB020206	This study
C	Soil clone C0210	92 (366/397)	AF128659	15
D	Soil clone C113	94 (400/425)	AF013535	26
F	Marine sediment clone	90 (529/587)	AB015254	33
G	*Actinomadura aurantiaca*	94 (533/563)	AF134066	62
H	*Streptomyces neyagawaensis*	100 (568/568)	AJ399493	This study
I	*Lentzea albidocapillata*	99 (567/568)	X84321	27

[a] See Fig. 5. Sequences for bands A to I have been deposited in GenBank and given accession numbers AF543360 to AF543367.

TABLE 3. Identity and relative abundance of bacteria isolated from SCN cysts on actinomycete medium or NBYEA[a]

Accession no.	Closest relative (accession no., % similarity)	No. of isolates from[b]:		No. of REP fingerprints[c]	Reference
		AM	NBYEA		
AF547025	*Lysobacter antibioticus* (AB019582, 98)	36	19	7,4	12
AF547026	*Variovorax* P9G781 (AF214127, 99)	10	42	5,12	51
AF547027	*Variovorax* P16G917 (AF214129, 100)	11	27	7,6	51
AF547028	*Microbacterium trichotechnolyticum* (MTR17240, 100)	8	12	3,4	50
AF547029	*Ultramicrobacterium* sp. strain 12-3 (AB008507, 97)	11	4	2,2	21
AF547030	*Dyadobacter fermentans* (AF137029, 98)	13	2	5,2	7
AF547031	*Ensifer* sp. strain P21375 (AF214726, 99)	2	12	1	51
AF547032	*Rhizobium* sp. strain N220 (AF195069, 100)	10	2	2,1	61
AF547033	*Polaromonas vacuolata* (U14585, 98)	0	9	0,2	22
AF547034	*Agromyces cerinus* (X77448, 99)	2	7	1	44
AF547035	*Streptomyces neyagawaensis* (AJ399493, 100)	7	0	5	This study
AF547036	*Cytophaga* sp. strain D2 (AF250407, 98)	6	0	1	14
AF547037	*Sinorhizobium* sp. strain S002 (AF285962, 100)	5	0	3	9
AF547038	*Saccharothrix flava* (AF114808, 99)	4	0	3	28
AF547039	Bacterium P91635 (AF214121, 100)	3	0	1	51
AF547040	*Rhodococcus* sp. strain RHA7 (RSU16315, 100)	3	0	2	This study
AF547041	*Streptomyces thermocarboxydus* (STH249627, 99)	3	0	1	This study
AF547042	*Flexibacter sancti* (M62795, 94)	3	0	2	59
AF547043	Potato root clone (AJ252710, 99)	3	0	1	This study
AF547044	*Pseudomonas fluorescens* (AF134705, 99)	0	3	2	54
AF547045	Freshwater clone (AJ224987, 97)	0	2	1	17
AF547046	*Agromyces ramosus* (X77447, 98)	0	1	1	44
AF547047	*Streptomyces paradoxus* (SPA276570, 100)	1	0	1	This study
AF547048	*Streptomyces* sp. strain LS-1 (AF275257, 100)	1	0	1	This study
AF547049	*Streptomyces turgidiscabies* (AB026221, 100)	1	0	1	6
AF547050	*Saccharothrix tangerinus* (AB020031, 99)	1	0	1	This study
AF547051	*Williamsia muralis* (WMU17384, 99)	1	0	1	24
AF547052	*Pedobacter heparinus* (M11657, 99)	1	0	1	56
AF547053	Marine sediment clone (AB015566, 99)	1	0	1	32
AF547054	*Xanthomonas* sp. strain AK (AB016762, 97)	1	0	1	47

[a] A representative partial 16S rRNA gene sequence (positions 341 to 928) from each group has the indicated GenBank accession number. The closest relative identified by BLAST search of the GenBank is indicated, as well as the number of clones recovered from actinomycete medium (AM) or NBYEA, number of REP fingerprint patterns detected in the group on AM or NBYEA, and relevant citation.
[b] Total number of isolates from AM and NBYEA, 290.
[c] Total number of REP fingerprints, 97.

of morphological types detected, the level of diversity in this habitat was much less than that described for biofilms by Manz et al. (34) using the same approach. Lectin staining patterns in the matrix were heterogeneous and patchy in nature; however, it could not be confirmed whether specific lectin staining was associated with bacterial growth in the matrix material. Examination of the physical appearance of the interior material indicated that it consisted of numerous globular and spherical structures (Fig. 1B). Further analyses of the cysts indicated that the internal cyst material contained a limited range of glycoconjugates, while Nile red staining implied a lipid-rich hydrophobic nature. The binding of these lectins is suggestive of the presence of *N*-acetylglucosamine (*S. tuberosum*), α-D-galactosyl (*B. simplicifolia*), and α-D-mannosyl and glucosyl residues (*L. culinaris*).

A comparison of the direct and plate counts indicates that only a small fraction, less than 5%, of the total populations were culturable. There was little relationship between the dominant phylotypes detected by DGGE analysis and the collection of bacteria obtained by plating on actinomycete medium and NBYEA. This variance is to be expected because of culture bias. The actinomycete medium captured more diversity (26 phylotypes) than did NBYEA (13 phylotypes). Only four of the 13 phylotypes detected on NBYEA were not likewise detected on the actinomycete medium. Over half of the isolates were *Lysobacter* or *Variovorax* sp., which readily grew on both isolation media. Numerous REP fingerprints obtained for these dominant phylotypes indicate that there was significant clonal diversity. We are currently screening bacteria for antibacterial, antifungal, and nematocidal activities in order to gain insights into their possible relationship with SCN in soils and potential as biocontrol agents.

One of the more interesting findings of this study is the prominence of bacteria detected both in the DGGE analysis and upon isolation, which characteristically produce antimicrobial agents and polymer-hydrolyzing enzymes. A number of genera of actinobacteria were isolated from cysts, including *Streptomyces*, *Agromyces*, *Saccharothrix*, *Microbacterium*, and *Williamsia*. Members of these genera are well known to produce antibacterial and antifungal compounds and agents that are antineoplastic or otherwise cytotoxic. Also noteworthy is the presence within cysts of *Lysobacter antibioticus*, a gammaproteobacterium in the *Xanthomonas* group (12). Members of this genus are characteristically antibiotic producing, strongly proteolytic, degrade chitin, and can lyze bacteria, fungi and nematodes (11, 19, 39, 60). Some members of the *Rhizobiaceae* were represented in the collection, *Ensifer* sp., *Rhizobium* sp., *Sinorhizobium* sp., and bacterium P91635. We have not determined if these nodulate soybeans. All of the bacteria detected in this study can reasonably be expected to be soil residents,

with the possible exception of *Polaromonas vacuolata*, described as a psychrophilic, marine, gas vacuolate bacteria from Antarctica (22).

Given the abundance and viability of the cyst bacterial community, the cyst interior is clearly providing a suitable environment for the growth of these bacteria, presumably at the nematode's expense, and perhaps to its detriment. The potential significance of the bacterial flora within SCN cysts to the survival or pathogenicity of this obligately endoparasitic nematode is at this point entirely unknown, but a number of intriguing hypotheses can be conjectured.

Cysts in soils are parasitized by fungi and preyed upon by sprintails (*Collumbola*) (52). Production of chemical agents by the cyst's bacterial complement which repel or inhibit such organisms could serve to protect the nematode eggs in this hostile environment.

The eggshell of nematodes in the genus *Heterodera* is constructed of various polymers, collagen-like proteins, chitin, and lipoproteins, organized in multiple layers (23). Bacterial production of polymer-hydrolyzing enzymes within the cyst could decrease the rigidity of the eggshell, facilitating the exit of the emerging juvenile (42). Quiescent juveniles within eggs can be induced to hatch by exposure to host plant root exudates, synchronizing the emergence of nematodes with the presence of the host (23). Cyst bacteria could mediate or interfere with this process, by transforming these hatch-inducing substances or by themselves producing hatch-inducing substances.

In conclusion, this study has shown that the senescent resting structure of this economically important crop pest is associated with relatively undiverse bacterial communities and that some bacteria are found in all communities suggesting some relationship with SCN. Further studies will consist of establishing the function of bacteria isolated from cysts in SCN pathogenicity or control and examining the microbial complement of this nematode throughout its life cycle.

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