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Long-Term Exposure of Agricultural Soil to Veterinary Antibiotics Changes the Population Structure of Symbiotic Nitrogen-Fixing Rhizobacteria Occupying Nodules of Soybeans (*Glycine max*)

Cécile Revellin,[a] Alain Hartmann,[a] Sébastien Solanas,[a] Edward Topp[b,c]

[a]Agroécologie, AgroSup Dijon, INRA, University of Bourgogne Franche-Comté, Dijon, France
[b]Agriculture and Agri-Food Canada, London, ON, Canada
[c]University of Western Ontario, London, ON, Canada

ABSTRACT Antibiotics are entrained in agricultural soil through the application of manures from medicated animals. In the present study, a series of small field plots was established in 1999 that receive annual spring applications of a mixture of tylosin, sulfamethazine, and chlortetracycline at concentrations ranging from 0.1 to 10 mg · kg^{-1} soil. These antibiotics are commonly used in commercial swine production. The field plots were cropped continuously for soybeans, and in 2012, after 14 annual antibiotic applications, the nodules from soybean roots were sampled and the occupying bradyrhizobia were characterized. Nodules and isolates were serotyped, and isolates were distinguished using 16S rRNA gene and 16S to 23S rRNA gene intergenic spacer region sequencing, multilocus sequence typing, and RSα fingerprinting. Treatment with the antibiotic mixture skewed the population of bradyrhizobia dominating the nodule occupancy, with a significantly larger proportion of *Bradyrhizobium liaoningense* organisms even at the lowest dose of 0.1 mg · kg^{-1} soil. Likewise, all doses of antibiotics altered the distribution of RSα fingerprint types. Bradyrhizobia were phenotypically evaluated for their sensitivity to the antibiotics, and there was no association between *in situ* treatment and a decreased sensitivity to the drugs. Overall, long-term exposure to the antibiotic mixture altered the composition of bradyrhizobial populations occupying nitrogen-fixing nodules, apparently through an indirect effect not associated with the sensitivity to the drugs. Further work evaluating agronomic impacts is warranted.

IMPORTANCE Antibiotics are entrained in agricultural soil through the application of animal or human waste or by irrigation with reused wastewater. Soybeans obtain nitrogen through symbiotic nitrogen fixation. Here, we evaluated the impact of 14 annual exposures to antibiotics commonly used in swine production on the distribution of bradyrhizobia occupying nitrogen-fixing nodules on soybean roots in a long-term field experiment. By means of various sequencing and genomic fingerprinting techniques, the repeated exposure to a mixture of tylosin, sulfamethazine, and chlortetracycline each at a nominal soil concentration of 0.1 mg · kg^{-1} soil was found to modify the diversity and identity of bradyrhizobia occupying the nodules. Nodule occupancy was not associated with the level of sensitivity to the antibiotics, indicating that the observed effects were not due to the direct toxicity of the antibiotics on bradyrhizobia. Altogether, these results indicate the potential for long-term impacts of antibiotics on this agronomically important symbiosis.

KEYWORDS nitrogen fixation, postantibiotic effect, soil microbiology, soybean

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Address correspondence to Edward Topp, ed.topp@agr.gc.ca.

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In commercial North American and European agriculture, antibiotic medicines are introduced into agricultural soil through normal farming practice (1). Some antibiotics are used for controlling bacterial diseases of some crops, for example, streptomycin or tetracycline to control the loss of production from fruit trees due to infection of the blossoms with *Erwinia amylovora* (2). Manure from medicated animals either applied as a fertilizer or excreted directly on the pasture will contain drug residues that are excreted intact (1–7). Antibiotics that are not degraded during the treatment of municipal sewage and that sorb to organic matter will partition into the recovered sludge (8–10). Many jurisdictions permit the beneficial reuse of sewage sludge (biosolids) as a soil fertilizer and amendment, and this practice will entrain antibiotics in soil (8, 11). Finally, the irrigation of crops with reclaimed effluent from municipal wastewater treatment plants will likewise introduce antibiotics into the soil (12–15). Overall, soils that receive agricultural or urban waste streams will be exposed to antibiotics.

The potential impact of antibiotics on environmental bacteria is of significant concern, both from the perspective of enhancing the environmental reservoir of antibiotic resistance (the resistome) and through the inhibition of microorganisms that carry out important ecosystem services (16–22). Symbiotic nitrogen fixation is very important in commercial crop production, reducing the economic costs and the environmental concerns associated with the use of inorganic nitrogen fertilizers (23). Soybean (*Glycine max* [L.] Merr) is globally the most important source of plant-based protein for animal feed and other applications and represents approximately 50% of the total global acreage cropped for legumes with an estimated annual production of 315 megatons from 2014 to 2015 (23–25). As with all other legumes, soybeans obtain nitrogen for crop growth through their association with symbiotic nitrogen-fixing rhizobacteria (26). At least five *Bradyrhizobium* species, namely, *B. japonicum*, *B. elkanii*, *B. liaoningense*, *B. diazoefficiens*, and *B. yuanmingense*, are known to nodulate and fix nitrogen in association with soybeans (27). The competitiveness and nitrogen fixing activity of different *Bradyrhizobium* species strains can vary significantly; therefore, the inoculation of soybean seeds with commercial strains of bradyrhizobia is often undertaken to ensure rates of nitrogen fixation that support high crop yields (28). The abundance and diversity of soil populations of *Rhizobium* or *Bradyrhizobium* can be impacted by the cropping sequence, the application of chemical fertilizers, by chemical pollutants or environmental stresses such as drought (29–32).

Within the context of evaluating the interactions of antibiotics with soil microorganisms, a long-term field experiment was initiated in London, Ontario, Canada, in 1999 to evaluate the impact of selected veterinary antibiotics on soil microorganisms (33, 34). Field plots receive an annual spring application of a mixture of tylosin, sulfamethazine, and chlortetracycline, simulating the exposure of soil to drugs typically carried in manure from commercial swine production. Since 1999, the plots have been cropped continuously for soybeans that have never been treated with inoculants; thus, bacteria occupying the nitrogen-fixing nodules will have originated from the endogenous soil community. In the present study, the identities and diversity of dominant culturable bradyrhizobia recovered from soybeans sown in 2012 following 14 annual applications of antibiotics were characterized. The specific objectives of the present study were to (i) determine if the diversity and dominant culturable types of bradyrhizobia occupying nitrogen-fixing nodules varied with long-term soil exposure to antibiotics, and if any effect was dose dependent, (ii) characterize the identities of the recovered bradyrhizobia, and (iii) determine if any treatment effect was associated with a variation in the susceptibility of the nodule-occupying bradyrhizobia to the applied antibiotics.

RESULTS

Strain isolation and identification of bradyrhizobia from nodules. A total of 382 nodules were processed for bacterial isolation. From these, 301 putative nodulating isolates were obtained and purified by spreading to individual colonies. All isolates were kept frozen at −80°C until further analysis. The isolates were tested for their capacity to nodulate soybeans in a laboratory bioassay. On this basis, a total of 281 isolates potentially nodulating soybeans were further analyzed for their phenotypic and

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TABLE 1 Distribution of bacterial serogroups observed in crushed nodules from soils exposed in the field to the indicated antibiotic concentrations[a]

Serogroup	% of isolates with soil antibiotic exposure (mg · kg^{-1} soil)			
	Control (0)	Low dose (0.1)	Intermediate dose (1.0)	High dose (10.0)
Serogroup 135	21.9	30.6	36.6	48.0
Other serogroups	13.5	21.4	14.9	14.3
No reaction	64.6	48.0	48.5	37.8

[a]The distributions of isolates in the three classes (serogroup 135, other serogroups, and no reaction) were significantly different among isolates obtained from the 4 soils with variable antibiotic exposure (χ^2 test observed value, 19.8; critical value, 12.6; 6 degrees of freedom; $P = 0.003$ with an α of 0.05).

genotypic characteristics. Similar numbers of isolates (56, 78, 79, and 68 isolates for control, low-dose, intermediate-dose, and high-dose soils, respectively) were obtained from soybean plants recovered from each of the antibiotic-treated and the control soils.

Phenotypic characterization of bradyrhizobia in crushed nodules and isolates of nodulating bradyrhizobia. Immunological reactions against the five antisera of the 382 nodules showed that the dominant serogroup among those bacteria that reacted with the antisera was serogroup 135 (antisera targeting *B. liaoningense* 2281^T) (Table 1). Other serogroups (i.e., 122, 6, 123, and 110) were rarely present and thus were pooled for the statistical analysis. A large pool of nodules did not react with any antisera and are designated "no reaction" (Table 1). A chi-square analysis of the data revealed that the distribution of nodule serotype varied significantly according to treatment ($P = 0.003$). A Fisher exact test revealed that the number of nodules occupied with serogroup 135 isolates was significantly higher in soil treated with the high dose (10 mg · kg^{-1}) of antibiotics than in the untreated control soil (Fisher exact test, $\alpha = 0.05$).

Antibiotic susceptibility of isolates. The susceptibility of the 272 isolates to each of the three antibiotics at 1, 10, and 100 mg · liter^{-1} was evaluated. There was no difference in the frequency of susceptibility to any of the antibiotics according to whether the isolates were obtained from soybeans grown in the absence or presence of antibiotics in the field experiment (Table 2). At the higher challenge concentration (100 mg · liter^{-1}), 30 to 60% of the isolates were resistant to sulfamethazine. The isolates were generally sensitive to 100 mg · liter^{-1} of either chlortetracycline or tylosin.

The distribution of isolates resistant to the three antibiotics together did not vary according to which treatment they were obtained from in the field experiment (Table 3). The majority of isolates (89%) belonging to serogroup 123 were resistant to the mixture of the three antibiotics regardless of which treatment they were obtained from (highly significant by chi-square analysis, $P > 0.0001$).

Genotypic diversity of isolates. The 275 isolates were subjected to RSα fingerprinting (Fig. 1). A total number of 21 distinct RSα fingerprints was observed. The

TABLE 2 Percentages of nodule isolates resistant to CTC, SMZ, and TYL from soils having received increasing concentrations of antibiotics

Soil antibiotic treatment (mg · kg^{-1})	No. of isolates	% of resistant isolates in culture medium containing (mg · liter^{-1})[a]:								
		CTC[b] (1)	CTC (10)	CTC (100)	SMZ[c] (1)	SMZ (10)	SMZ (100)	TYL[d] (1)	TYL (10)	TYL (100)
Control (0)	51	100.0	97.2	10.9	100.0	100.0	60.8	100.0	71.9 A	6.2
Low dose (0.1)	78	98.8	98.8	16.7	84.0	79.0	33.6	96.3	49.1 AB	2.9
Intermediate dose (1)	79	98.8	91.2	15.5	91.7	88.1	33.4	100.0	47.3 AB	3.7
High dose (10)	64	98.6	76.8	1.4	100.0	98.6	36.1	90.3	31.1 B	3.0
Pr > *F*		0.800	0.236	0.692	0.564	0.598	0.164	0.538	0.062	0.881
Statistical significance at *P* value of 0.05		NS[e]	NS	NS	NS	NS	NS	NS	S[f]	NS

[a]Data from each column were subjected to analysis of variance (ANOVA) followed by a Newman and Keuls test ($P = 0.05$) using XLSTAT-18.07 software (Addinsoft). Data followed by the same uppercase letter are not significantly different ($P > 0.05$).
[b]CTC, chlortetracycline.
[c]SMZ, sulfamethazine.
[d]TYL, tylosin.
[e]NS, no significant difference.
[f]S, significant difference.

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TABLE 3 Percentages of root-nodulating isolates resistant to a mixture of the three antibiotics (sulfamethazine, tylosin, and chlortetracycline) each supplemented into the culture medium at a concentration of 10 mg · liter^{-1}

Soil antibiotic treatment (mg · kg^{-1})	% of resistant isolates[a]			
	Mean	SE	Lower limit (95%)	Upper limit (95%)
Control (0)	54.0	9.8	31.3	76.7
Low dose (0.1)	61.0	9.8	38.3	83.7
Intermediate dose (1)	37.3	9.8	14.6	60.0
High dose (10)	50.3	9.8	27.6	73.1

[a]The isolates were obtained from plants grown in soils that received the indicated concentrations of antibiotics in the field. An ANOVA indicated the differences were not significant ($P = 0.434$).

distribution of isolates according to their RSα fingerprint among nodules obtained from the various soil treatments is given in Fig. 2. The distribution of isolate RSα fingerprints was significantly different between the control soil and the soils receiving antibiotics at any of the three application rates (chi-square analysis, P value = 0.000). A subset of 21 isolates (one per distinct RSα fingerprint) was further characterized by multilocus sequence type (MLST) analysis and 16S rRNA gene and intergenic spacer (IGS) sequencing (Fig. 3). Soybean-nodulating isolates were distributed within 9 sequence types (ST) and in the four *Bradyrhizobium* lineages, II, III, IV, and V (based on 16S rRNA gene and IGS sequencing). The MLST analysis confirmed that serogroup 135 isolates harboring RSα fingerprints a, b, c, and q are closely related to *B. liaoningense*; there were only one or two mismatches compared to the *B. liaoningense* sequence over the concatenated sequence of the six housekeeping genes analyzed. The proportion of soybean nodule isolates belonging to *B. liaoningense* species (isolates harboring RSα fingerprints a, b, c, and q) increased significantly after treating soils for 14 years with antibiotics (Fig. 2). On the other hand, the proportion of isolates harboring RSα fingerprints p, r, and s decreased in nodules obtained from plants grown in soils that received antibiotics.

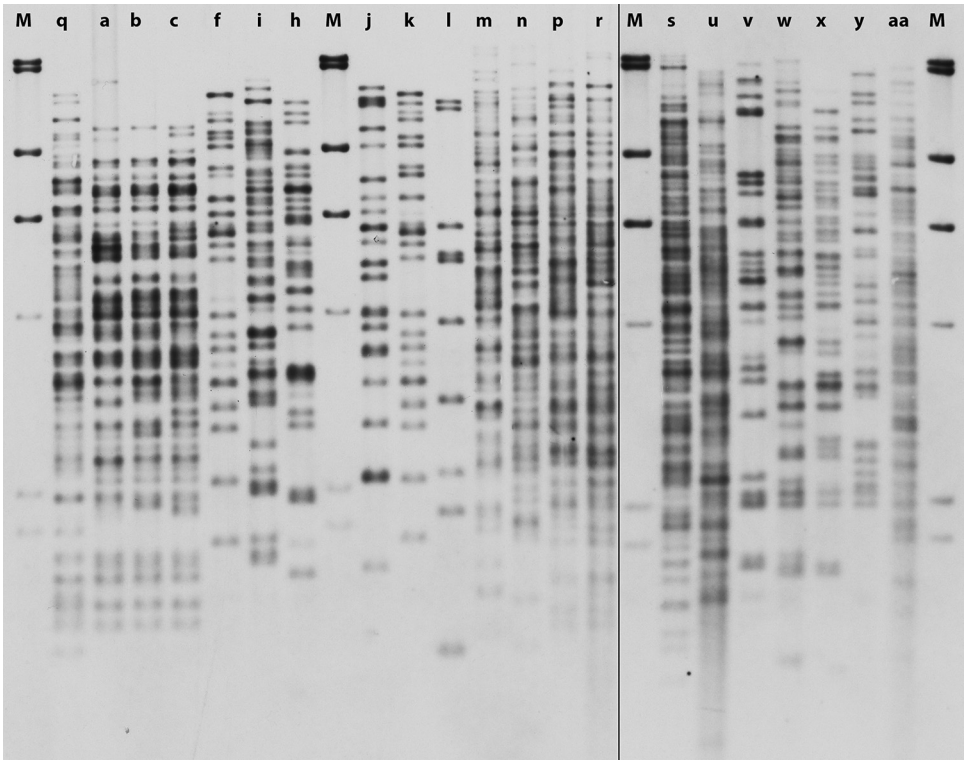

FIG 1 RSα fingerprints of representative soybean-nodulating isolates. Lanes are labeled with letters specifying fingerprint identifiers; M indicates the DIG-labeled molecular weight marker II (Roche, France).

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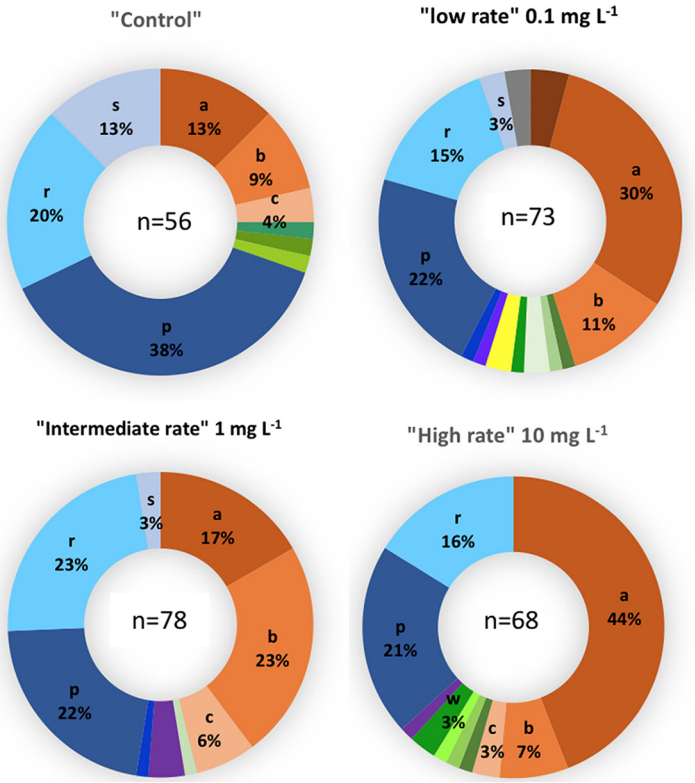

FIG 2 Distributions of RSα fingerprints of isolates obtained from plants grown in plots receiving the indicated antibiotic treatments. The numbers of distinct RSα profiles for the control, low-dose, intermediate-dose, and high-dose treatments were 9, 14, 9, and 10, respectively.

DISCUSSION

Although nodules were collected in Canada and dehydrated and transported to France for further strain isolation, we were able to successfully isolate 281 strains from 382 nodules. The small size of certain nodules as well as the dehydration-rehydration procedure of nodules and the occurrence of fast growing contaminants might explain the fact that some isolates were not recovered. This is frequently the case in such studies (C. Revellin, personal observation). Serological identification was primarily done on crushed nodules and was in good agreement with the serological identification of isolates (data not shown). We were able to further characterize a total of 272 and 275 isolates by phenotypic and genotypic methods, respectively. A few isolates (9 and 6) were eliminated either because they were not able to grow again in culture or because we were unable to obtain good quality DNA for their genotypic characterization.

A major finding of this study is that repeated annual drug applications to soil impacted the structure of the *Bradyrhizobium* populations recovered from soybean nodules. Referenced to that of control soil not receiving antibiotics, the composition of bradyrhizobial populations occupying soybean nodules was significantly different following 14 annual applications of a mixture of veterinary drugs. Of note was the higher proportion of *B. liaoningense* detected in nodules of soybeans grown in antibiotic-treated soils. This was clearly demonstrated on crushed nodules, where the proportion of serogroup 135 isolates increased significantly in soils having received the highest dose of antibiotics compared to that in control soil. Serogroup 135 isolates could be further assigned to *B. liaoningense* species by a genotypic analysis. Indeed, 16S rRNA gene and IGS sequencing of four isolates belonging to serogroup 135 revealed a nearly 100% identity (only one mismatch in IGS sequence for RSα type c isolates) with the corresponding sequences of *B. liaoningense* strain 2281^T. RSα fingerprinting was more discriminant than serotyping and enabled the characterization of isolates which did not

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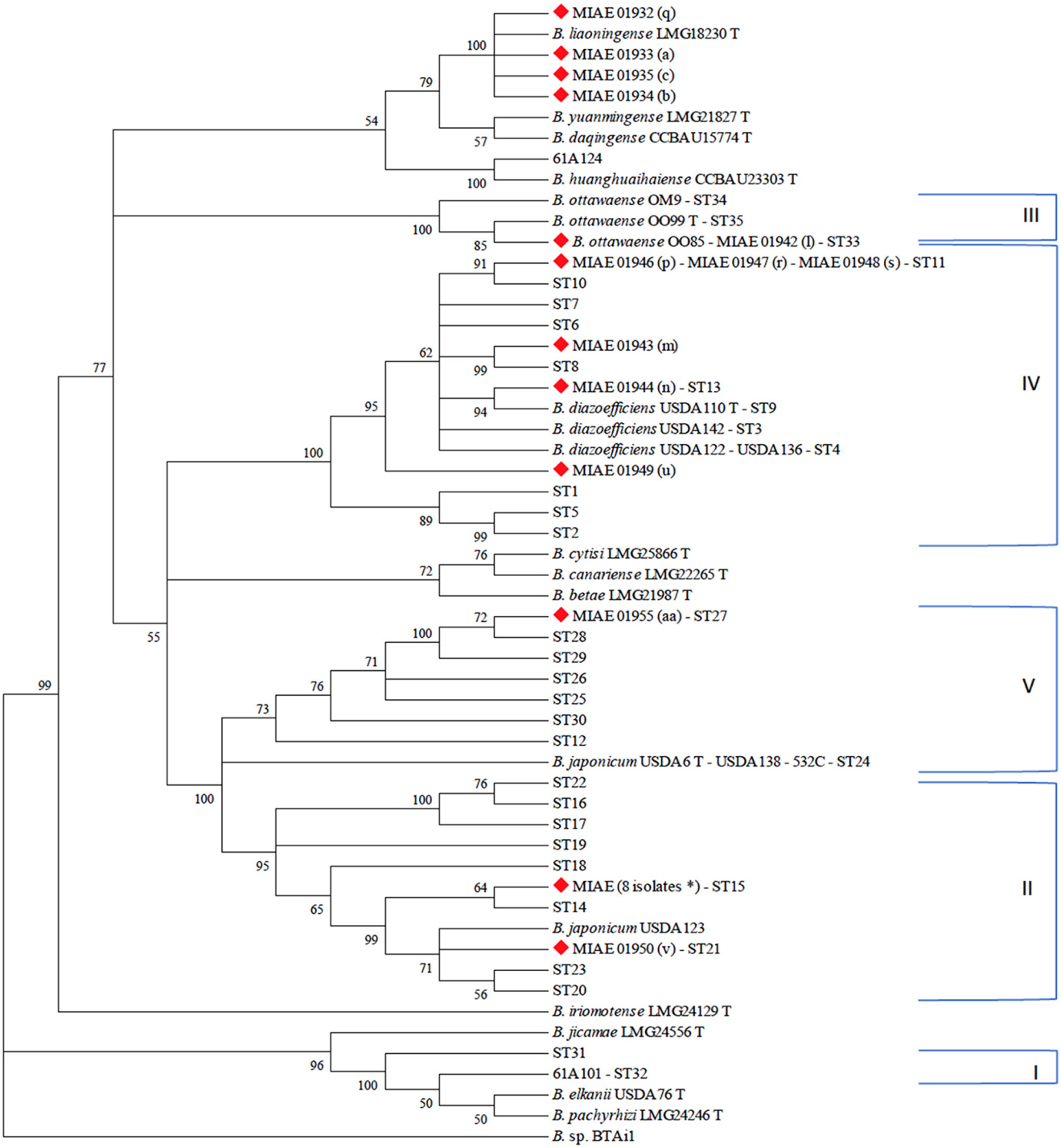

FIG 3 Molecular phylogenetic analysis by maximum likelihood method of the 21 soybean-nodulating isolates based on MLST analysis (concatenated gene sequences). Roman numbers indicates *Bradyrhizobium* lineages as described by Tang et al. (27). Sequence types (STs) defined by Rivas et al. (54) are indicated. Type strains of *Bradyrhizobium* species are indicated by "T"s. Isolates of this study are identified by red diamonds, their collection numbers (MIAE), and their RSα types. "8 isolates *" belonging to ST15: MIAE 01937 (f), MIAE 01938 (h), MIAE 01939 (i), MIAE 01940 (j), MIAE 01941 (k), MIAE 01951 (w), MIAE 01952 (x), MIAE 01953 (y). The evolutionary history was inferred by using the maximum likelihood method based on the general time reversible model. The bootstrap consensus tree was inferred from 1,000 replicates. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed.

react to any serum. Isolates from serogroup 135 (*B. liaoningense* species) harbored four different RSα fingerprints, namely, a, b, c, and q. The distribution of isolates in RSα fingerprints was significantly different in soils treated with antibiotics compared to that in control soil, with an increase in the proportion of strains belonging to RSα types a,

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b, c, and q and a decrease in strains belonging to RSα types p and s. This result confirms those based on serotyping. RSα fingerprints tended to be more diverse in soils having received antibiotics compared to that in control soils. Shannon diversity indexes computed for control and antibiotic- treated soils were equal to 1.11, 1.53, 1.43, and 1.3 for the control soil and soils having received low, intermediate, and high antibiotic doses, respectively. A *t* test revealed that Shannon diversity indexes were significantly different between the control soil and soil that received the low dose of antibiotics ($P = 0.0045$). Even the low dose of antibiotics applied to soil increased the diversity of soybean-nodulating populations. Higher antibiotic concentrations did not appear to produce the same significant effect. This might be due to competition issues between dominant and less abundant nodulating strains in soils that could be impacted by antibiotics. The reduction in the number of dominant strains might enable nodulation by less abundant or less competitive strains.

The most facile explanation for the observed effects was that bradyrhizobia that were less susceptible to the antibiotics had a competitive advantage in antibiotic-treated soils and would therefore be overrepresented in nodules from plants grown in the plots treated with antibiotics. The antibiotic susceptibility testing of the isolates indicated that this was not the case (Tables 2 and 3), no doubt because of the general intrinsic resistance of bradyrhizobia to antibiotics (35). If anything, *B. liaoningense*, prominent in nodules from treated plots, was more susceptible to the antibiotics used than the other isolates. What other potential effects of the antibiotic treatments could then explain the observation? One hypothesis is that the antibiotics were acting on the soybean plants, altering their interaction with bradyrhizobia in the establishment of the nitrogen-fixing symbiosis. Numerous classes of antibiotics have the potential to inhibit plants, largely through the bacterial ancestry of chloroplasts and mitochondria. For example, sulfadiazine inhibits the root elongations of wheat (*Triticum aestivum* L.), Chinese cabbage (*Brassica campestris* L.), and tomato (*Cyphomandra betacea*), with 50% inhibitory concentrations (IC_{50}s) ranging from 28 to 93 mg · kg^{-1} soil (36). In a hydroponic screening assay, chlortetracycline, sulfamethoxazole, and tylosin significantly inhibited the root elongation of carrots (*Daucus carota*), with 50% effective concentrations (EC_{50}s) of 1,141, 60, and 542 μg · $liter^{-1}$, respectively (37). In the present study, there was no obvious inhibition of seed germination or plant growth on the basis of the numbers of surviving plants, the delay in emergence, or visually obvious differences in yield. We have no observations of root biomass or architecture and therefore cannot comment on whether or not the below-ground portion of the plant varied morphologically according to the antibiotic treatments. We note that the half-lives for the antibiotics in soils from these plots were in the range of 1 to 10 days (34) and that the treatment effect was observed at the lowest dose of 0.1 mg · kg^{-1} soil. Thus, the exposure time was short relative to the growing season, and the exposure concentration was low relative to published toxicological endpoints (36, 37). Nevertheless, we cannot discount a phytotoxic effect, particularly given that the treatment consisted of antibiotic mixtures with unknown mixture interactions. A final hypothesis would be that some effect of the antibiotic treatment on components of the soil microbiome other than the bradyrhizobia indirectly resulted in the various nodule occupancies observed here. In these same soils, the high-dose exposure significantly modified global soil communities as revealed through metagenomic inventories (33). Numerous classes of antibiotics can alter the expression of genes involved in nutrient cycling, communication, and other critically important ecosystem processes at subclinical concentrations (20, 38, 39). Some bacteria, for example cyanobacteria, are sensitive to antibiotics at concentrations far below clinically relevant MICs (40). Erythromycin at concentrations of 1 to 4 μg · $liter^{-1}$ perturbed aquatic biofilm structure and function (41). Overall, given the critical ecosystem services that microorganisms undertake, gaining further insight into their interactions with antibiotics entrained in soil through the application of animal manures, sewage sludge, or recycled wastewater is a clear research priority with respect to defining the sustainability of these practices (20, 42, 43).

Given the dominant global value of this crop and the immensely important eco-

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system service that symbiotic nitrogen fixation represents, the practical agronomic significance of this result merits further investigation. The experimental format which employed very small field plots and added drugs directly to soil without the application of animal manure obviously did not represent normal farming practice. It would be of value to determine if bradyrhizobial species differ significantly in field soils receiving manures from medicated animals compared to those in soils fertilized with manures from animals that are produced without antibiotic use. Likewise, the competitive abilities and nitrogen-fixation efficiencies of nodule occupants grown in the presence and absence of antibiotic exposure would be important to determine. Finally, it may be worth confirming the assumption that low residual antibiotic concentrations would not reduce the nitrogen-fixing success of soybean crops heavily amended with commercial inoculants as is commonly practiced in Europe and North America.

Overall, the present study indicates that long-term soil exposure with environmentally relevant concentrations of a mixture of the veterinary antibiotics tylosin, chlortetracycline, and sulfamethazine alters the composition of bradyrhizobial populations occupying soybean nodules. From the perspective of an ecotoxicology evaluation of long-term antibiotic effects, the interaction of legumes with their symbiotic nitrogen-fixing partners is a highly interesting endpoint. Further work evaluating the potential impacts of antibiotics carried in animal manures applied according to normal farming practice on crop yield and nitrogen fixation rates is called for.

MATERIALS AND METHODS

Field operations and nodule sampling. The field experiment was described in detail in Topp et al. (34). Briefly, a series of replicated 2-m^2 plots on the Agriculture and Agri-Food Canada research farm in London, Ontario, received annual spring applications of a mixture of tylosin, sulfamethazine, and chlortetracycline, antibiotics commonly used in commercial swine production. From 1999 to 2004, the nominal drug concentrations at application were 0.01, 0.1, and 1.0 mg · kg^{-1} soil to a depth of 15 cm. Starting in 2005, the drug concentrations were increased 10-fold to 0.1, 1.0, and 10 mg · kg^{-1} soil. Each mixture concentration was applied to triplicate plots, and in addition, three plots remained untreated as controls. Each June, the antibiotics were applied by removing 1-kg portions of soil from each plot, amending these with aqueous solutions of the antibiotics, and uniformly incorporating the amended soil to a depth of 15 cm with a mechanical rototiller. Plots were continuously cropped for soybeans (*Glycine max* var. Harosoy) and received no further management other than the removal of weeds by hand. The soybean seeds for this experiment were propagated annually on the Agriculture and Agri-Food Canada (AAFC) research farm and were never treated with a commercial inoculant. All plots were seeded by hand each June within 48 h of the antibiotic application, a process that took less than 1 h. Nodule samples were collected during the second week of August 2012, the plots in that year having received 14 annual applications of antibiotics. Five nodulated plants were collected at random from each plot, and from each of the plants, 5 to 7 nodules were excised at random. The numbers of nodules per sampled plant were not visibly different across treatments. Likewise, the above-ground plant biomasses were not visibly different across treatments, but the plots were too small to obtain any meaningful yield measurements.

Isolation of rhizobia from nodules. Nodules were dried in the presence of anhydrous $CaCl_2$, and then shipped from Canada to the INRA Agroecology laboratory in Dijon, France. The nodules were rehydrated in sterile water for 1 h, and then surface sterilized by soaking for 30 s in a freshly prepared saturated aqueous solution of $Ca(ClO)_2$. The nodules were then rapidly rinsed 5 times with 4 ml of sterile water and finally rinsed 5 times for 15 min each in 4 ml of sterile water. The surface-sterilized nodules were deposited in 96-well polystyrene microplates (Nunc, Dutscher, France) (one nodule per well) in 100 μl of saline water (8.5 g NaCl · $liter^{-1}$). The nodules were then thoroughly crushed with sterile wooden sticks. The isolation of rhizobia was performed from as described by Vincent (44) on modified Bergersen medium plates as described below. After isolation, the crushed nodule suspensions were kept frozen at −20°C as described previously (45), pending immunological characterization.

Bacterial strains and culture conditions. *Bradyrhizobium* strains, *B. liaoningense* 2281^T, and all isolates obtained during this study were grown in Bergersen medium (46) modified by removing the vitamins and supplementing with 0.2 g of yeast extract per liter. The isolation and purification (two or three times) of nodule isolates were primarily done by spread plating on modified Bergersen agar. Plates were incubated for 7 to 10 days at 28°C until well-isolated colonies reached 1 to 2 mm in diameter. *Bradyrhizobium* organisms were grown in liquid cultures on Cliquet liquid medium (47) supplemented with 5 g of glucose · $liter^{-1}$ and incubated for 5 to 7 days at 28°C with orbital shaking (150 rpm). Dense cell suspensions prepared in modified Bergersen liquid medium containing 12.5% glycerol were prepared for long-term storage at −80°C.

Nodulation test. Surface-sterilized soybean seeds were germinated in sterile perlite kept moist with deionized water and then grown aseptically in a Jensen tube assembly (44). Four-day-old plants were inoculated with 1 ml of a diluted suspension (10^8 CFU · ml^{-1}) of each bacterial isolate to be tested. The presence of nodules on test plants was recorded after incubation for one and a half months in a temperature-controlled cabinet set at 20°C for 14 h/day and at 18°C for 10 h/night.

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Serogroup determination. Rabbit polyclonal antisera were raised against *B. japonicum* strains MSDJG49, IRATSA1, USDA 110, and USDA 123, which belong to serogroups 122, 6, 110, and 123, respectively, and against *B. liaoningense* strain 2281^T, which belongs to serogroup 135 (48). These sera were produced by rabbits immunized with the given strains at IUT Claude Bernard University Lyon, France. Briefly, heat-inactivated bradyrhizobia were used to immunize rabbits, from which sera were prepared by coagulation and centrifugation. Raw serum titers were determined and sera were used at dilutions of 1/10,000 (sera 110 and 123) or 1/100,000 (sera 6, 122, and 135). Sera were used for nodule strain typing according to the method of Revellin et al. (45). Briefly, a 5-μl aliquot of each crushed nodule suspension was transferred to a new microplate and diluted with 200 μl of Tris-buffered saline (pH 7.5; 2 mM Tris and 500 mM NaCl). After mixing, 34 μl of the resulting suspension was applied to nylon membranes for immunoblotting (45).

Antibiotic susceptibility testing. Isolates were grown on Cliquet liquid medium as described above. After measurements of optical density at 600 nm (OD_{600}), the aliquots of the cultures were transferred to microtiter plates (Nunc) and diluted in sterile water to a cell density of approximately 10^6 cells · ml^{-1}. Five-microliter droplets of each cell suspension were then deposited (using a 48-tip multiple inoculator [Sigma-Aldrich, France]) on different plates of modified Bergersen agar medium supplemented or not with the various antibiotics as described below. All isolates were tested on Bergersen agar supplemented individually with each antibiotic (tylosin, chlortetracycline, and sulfamethazine) at three concentrations (1, 10, and 100 mg · liter^{-1}). A control plate (Bergersen without antibiotic) was also inoculated with each isolate. In a second experiment, all isolates were tested on Bergersen agar medium supplemented with a mixture of the three antibiotics, each one provided at concentrations of 0.1, 1, and 10 mg · liter^{-1} corresponding to the nominal concentrations in the field experiment. All plates were incubated for 4 to 5 days at 28°C, and the plates were then photographed using a Scan 300 instrument (Interscience, Paris, France). Image analysis was performed using the Bio 1D++ software for OD analysis (Vilber Lourmat, France). The growth of the isolates in the presence of antibiotics was estimated by a comparison with the growth observed on antibiotic-free control plates. The isolates showing growth on medium supplemented with the highest concentration of antibiotic, corresponding to 75 to 100% of the growth observed on control plates, were considered to be resistant to the antibiotic (at 100 mg · liter^{-1}) or to the mixture of antibiotics (with each antibiotic at 10 mg · liter^{-1}).

RSα fingerprinting. RSα is a reiterated sequence, which has the structure of an insertion sequence (transposase motif and inverted repeats), and is present in most *Bradyrhizobium* spp. that nodulate soybeans (49). However, its transposition has never been demonstrated, and RSα fingerprinting will successfully genotype *Bradyrhizobium* isolates with a high discrimination power (50, 51). Genomic DNA from soybean-nodulating isolates was prepared as described by Hahn and Hennecke (52) and digested with the restriction endonuclease XhoI (Roche, Sigma-Aldrich, France). Restriction fragments were separated on a 0.9% agarose (Sigma type II medium EEQ) gel in Tris-acetate-EDTA (TAE) buffer. The digoxigenin (DIG)-labeled DNA molecular weight marker II (Roche, Sigma-Aldrich, France) was also loaded on each gel. DNA fragments were then transferred to a nylon membrane (Nytran NY13; Sigma-Aldrich) using a VacuGene apparatus (Sigma-Aldrich). The membranes were then washed in 5× sodium saline citrate (SSC) and air dried. DNA was cross-linked to the membrane by UV irradiation for 3 min. The plasmid pRJ4060 (51), containing a 2.4-kb insert of *B. japonicum* strain USDA 110, was labeled by PCR amplification using a deoxynucleoside triphosphate (dNTP) mix containing alkali-labile digoxigenin-11-dUTP (Roche, Sigma-Aldrich, France). Hybridization was carried out under highly stringent conditions; posthybridization stringency washes were performed twice in 2× SSC and a 0.1% SDS solution for 5 min at room temperature and twice in 0.1× SSC and a 0.1% SDS solution for 15 min at 68°C. The detection of the hybridized probe was realized using a DIG luminescence detection kit (Sigma-Aldrich) and CDP-Star as the chemiluminescent substrate according to the Roche application manual. Lumi-film chemiluminescent detection film (Sigma-Aldrich) was used to detect and record the chemiluminescent signal. The films were then scanned to obtain pictures in order to analyze and group profiles.

16S rRNA gene and IGS sequencing and MLST analysis. A representative set of isolates consisting of type strains of the various *Bradyrhizobium* spp., as well as one isolate chosen from each RSα profile (n = 34), was characterized by amplifying the 16S rRNA gene coding region and the 16S rRNA to 23S rRNA gene intergenic spacer (IGS) using universal primer pairs 27F (5′-AGA GTT TGA TC[A/C] TGG CTC AG-3′)/1492R (5′-TAC GG[A/T/C] TAC CTT GTT ACG ACT T-3′) and 72F (5′-TGC GGC TGG ATC ACC TCC TT-3′)/38R (5′-CCG GGT TTC CCC ATT CGG-3′), respectively. The PCR products were checked for size and purity by agarose gel electrophoresis and subjected to Sanger sequencing (Beckman Coulter Genomics, France). The alignments were conducted using blastn software (NCBI). The 21 representative nodule isolates were subjected to MLST analysis as described by Yu et al. (53) and others (27, 54). Briefly, six housekeeping genes (*atpD*, *glnII*, *recA*, *gyrB*, *rpoB*, and *dnaK*) were amplified and sequenced, and the corresponding concatenated gene sequence (3,210 bp) thus produced was aligned with known sequences to define sequence types (ST) using ClustalW; distances and tree were computed using MEGA7. The sequence types were numbered as previously described (53, 54).

Accession number(s). The sequences obtained from MLST analysis were deposited in GenBank under the following accession numbers: *atpD*, accession numbers MF977518 to MF977540; *dnaK*, accession numbers MG014242 to MG014264; *gyrB*, accession numbers MG014265 to MG014287; *glnII*, accession numbers MG014288 to MG014310; *recA*, accession numbers MG014311 to MG014333; and *rpoB*, accession numbers MG014334 to MG014356.

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