

Diversity within Serogroups of *Rhizobium leguminosarum* biovar *viceae* in the Palouse Region of Eastern Washington as Indicated by Plasmid Profiles, Intrinsic Antibiotic Resistance, and Topography

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Serology, plasmid profiles, and intrinsic antibiotic resistance (IAR) were determined for 192 isolates of *Rhizobium leguminosarum* biovar *viceae* from nodules of peas (*Pisum sativum* L.) grown on the south slope and bottomland topographic positions in eastern Washington State. A total of 3 serogroups and 18 plasmid profile groups were identified. Nearly all isolates within each plasmid profile group were specific for one of the three serogroups. Cluster analysis of IAR data showed that individual clusters were dominated by one serogroup and by one or two plasmid profile groups. Plasmid profile analysis and IAR analysis grouped 72% of the isolates similarly. Most plasmid profile groups and several IAR clusters favored either the south slope or the bottomland topographic position. These findings show that certain intraserogroup strains possess a greater competitiveness for nodulation and/or possess a greater ability to survive in adjacent soil environments.

The competitiveness of *Rhizobium* and *Bradyrhizobium* inoculant strains for nodule occupancy has been widely studied to improve legume yields and to understand rhizobial ecology (1, 22, 24, 46). Serological techniques, in particular fluorescent antibodies, have been extremely useful in defining which strains occupy plant nodules. Members of a population exhibiting an identical antibody-antigen reaction are assigned to a serogroup and can be considered similar, if not identical, organisms. However, variability within serogroups has been demonstrated with immunodiffusion (20), plasmid profile analysis (23), symbiotic plasmid restriction analysis (50), protein profiles (16, 27, 30, 43), intrinsic antibiotic resistance (IAR) (3, 31, 52), bacteriophage typing (32, 43), and symbiotic effectiveness (16, 20, 50, 52). In sum, these studies have suggested that intraserogroup diversity may reflect important differences between isolates and/or strains.

A well-studied field environment in the Palouse region of eastern Washington State was selected to characterize the diversity within the two dominant serogroups of the region. Previous field studies demonstrated that serogroup II isolates dominate the nodules of peas on the dry, warm soil microclimate of the south slopes and ridgetops, while serogroup I isolates are preferentially recovered from nodules on the relatively moist and cool microclimate of the north slopes and bottomlands (36, 37). The role of soil microclimate in nodule serogroup distribution was supported in greenhouse studies by preconditioning bottomland and south slope soils to extremes in water potential prior to planting peas at field-capacity water potential. The proportion of rhizobia occupying the nodules shifted toward serogroup II in the dry preconditioned soil and toward serogroup I in the wet preconditioned soil (52). Furthermore, in sterile field soil the serogroup II type strain was shown to be more tolerant of low soil water potentials than was the serogroup I type strain, as observed by most-probable-number plant infectiv-

ity tests (38). Since soil water potential affects rhizobial nodule occupancy and survival at the serogroup level, the distribution of intraserogroup strains may also be affected by topographically determined differences in soil microclimate.

In our study, two methodologies were used to identify and characterize intraserogroup diversity. Plasmid profile analysis was deemed important because many important traits, including host specificity (28), nodulation (28), nitrogen fixation (44), competitiveness of nodule occupancy (7), bacteriocin production (25), and hydrogen recycling (5), are encoded by *Rhizobium leguminosarum* plasmids. Plasmid profiles have been used by other investigators to discriminate between field strains (9, 21, 23, 42, 51). The second methodology, IAR, tests the ability of isolates to grow on a large number of (individual) antibiotics present at a low concentration in the medium (3). Thus, IAR produces a detailed "fingerprint" of the organism. Together the methodologies should provide a highly specific definition of a strain, allowing a more in-depth examination of the relationship between soil microclimate and rhizobial diversity.

The objectives of this study were to (i) characterize and determine the relationships between a large number of isolates from field-grown pea nodules by use of serology, plasmid profile analysis, and IAR and (ii) determine if topographic position affects the distribution of plasmid profile groups (hereafter referred to as plasmid groups) or IAR clusters.

MATERIALS AND METHODS

Field isolation. A total of 192 isolates of *R. leguminosarum* biovar *viceae* were obtained on 12 June 1986 from field peas (*Pisum sativum* cv. Latah) on the bottomland and south slope topographic positions in eastern Washington 25 km north of Pullman. The south slope sampling area (30% slope) was located 110 m upslope from the bottomland area, in the same vicinity as that sampled in earlier studies (37, 38, 52). Two plants were sampled at 12-m intervals across a 48- by 48-m grid at each sampling area. Roots were washed with water, and two nodules were excised from each plant approximately 10 cm below ground level. Nodules were

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surface sterilized for 3 min in 2.6% hypochlorite and 0.04% Tween 80 surfactant, washed three times in sterile distilled water, and individually placed in wells of a sterile 96-well microdilution tray. Sterile saline (100 μ l; 0.85% [wt/vol] NaCl) was added to each well, and the nodules were crushed with a sterilized 96-prong multiple replicator that also transferred crushed nodule material to yeast extract-mannitol agar (54) containing 100 μ g of cycloheximide ml^{-1} . Isolates were purified on streak plates, and a single colony was selected for each isolate. Pure cultures were grown to the mid-log phase in yeast extract-mannitol broth, mixed 1:1 with 40% (wt/vol) glycerol, and stored at -70°C in cryotubes. These stock cultures were used in all studies to minimize potential changes in expression with multiple culturing and to prevent potential rearrangement of plasmids.

Serogroup determination. Antisera were prepared (53) to type strains C4202, M344, and C1204, representing serogroups I, II, and III, respectively (R. L. Mahler, personal communication). Agglutination response curves were constructed to define the optimal antiserum concentration. Stationary-phase cultures were grown on yeast extract-mannitol broth, pelleted (1-ml volumes centrifuged for 3 min at room temperature on a Microfuge E; Beckman Instruments, Inc., Fullerton, Calif.), and washed twice with sterile saline, and 100 μ l (2×10^8 cells) of resuspended cells was added to wells of microdilution trays containing antisera. A twofold dilution series was performed to result in final antiserum-saline dilutions of 1:45 to 1:1,440. Trays were placed in a 52°C water bath for 1 h and refrigerated at 4°C for 6 h, and agglutination was assessed visually by use of a colony plate counter. For removal of the weak cross-reaction of the M344 antisera, stationary-phase C4202 cells (20 ml) were pelleted, washed twice with saline, and repelleted, 3 ml of M344 antisera was added, and the pellet was disrupted by vortexing. The antiserum-cell suspension was incubated for 3 h at 37°C , the cells were removed by centrifugation, and the antisera were carefully decanted. Serology of field isolates was determined as described above with 15 μ l (C4202 and M344) or 28 μ l (C1204) of a 1:40 dilution of antiserum-saline and 20 μ l of a saline (lacking antiserum) control added to separate microdilution wells. Fluorescein isothiocyanate-labeled antisera were prepared (47) to verify the agglutination reactions of the 30 isolates of known serology and the 192 field isolates. Slides containing culture smears were reacted with the three fluorescein isothiocyanate-labeled antisera and viewed with a Zeiss epifluorescence microscope (47).

Plasmid profiles. Plasmid profiles were determined in 0.9% agarose gels by a modification of the Eckhart (17) rapid visualization technique as described previously (18). Two or more isolates showing an identical profile based on number and size of plasmids were defined as a plasmid group. Molecular weight standards were plasmids of *R. leguminosarum* T83K3 (48). The kilobase size of pJB5JI (8), contained in strain T83K3, was used to convert other plasmids of strain T83K3 to approximate kilobase sizes. Approximately 20% of the isolates were visualized on several occasions to ensure the reproducibility of plasmid profiles.

IAR. Glycerol stock cultures (2 μ l) were individually placed in wells of microdilution trays, and 100 μ l of sterile saline was added to each well. A 96-well multiple inoculating device was used to transfer 10 μ l (10^4 cells) to freshly prepared petri plates (150 by 15 mm) containing yeast extract-mannitol agar amended with antibiotic. Antibiotics (Sigma Chemical Co., St. Louis, Mo.) and concentrations were as follows (in micrograms milliliter^{-1}): carbenicillin

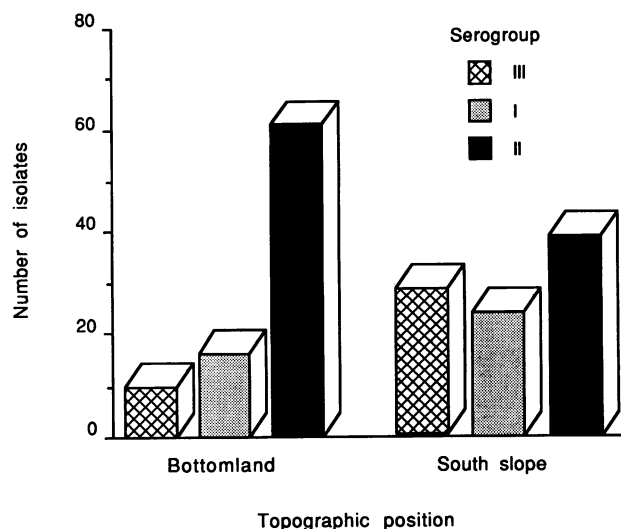


FIG. 1. Number of pea nodule isolates by topographic position and serogroup.

disodium salt, 20.0; chloramphenicol, 10.0; erythromycin, 2.0; kanamycin sulfate, 4.0; neomycin sulfate, 2.5; novobiocin sodium salt, 5.0; penicillin G sodium salt, 20.0; rifamycin SV sodium salt, 3.0; streptomycin sulfate, 5.0; tetracycline hydrochloride, 0.15; and vancomycin hydrochloride, 1.5. Previous experiments had established appropriate antibiotic levels. All isolates were plated in duplicate on each antibiotic medium. True intrinsic resistance was distinguished from a low frequency of spontaneous resistance by twice-daily inspections of the individual spots for developing confluent growth. Isolates were scored as sensitive or resistant after 3 to 4 days of growth at 27°C . Data were analyzed with the Biomedical Data Programs K means cluster analysis package (14). This program placed isolates with identical or very similar antibiotic reactions into an IAR cluster. The number of clusters present in the data set was determined by plotting Euclidean distance (the degree of scattering of isolates within a cluster around the mean value of the cluster) against number of clusters and maximizing for both low Euclidean distance and low cluster number. This program also indicated the significance of each antibiotic in defining individual IAR clusters and in defining all the IAR clusters collectively.

RESULTS

Antiserum specificity. The original antiserum titers were 2,560. A weak cross-reaction of the M344 antiserum with serogroup I isolates was removed by cross-adsorbing the antiserum with C4202 cells. The cross-adsorption did not affect the titer of the antiserum against homologous cells. Following cross-adsorption, 29 of 30 isolates of known serology reacted correctly in both agglutination and fluorescein isothiocyanate-labeled antiserum reactions. Agglutination reactions were verified with fluorescein isothiocyanate-labeled antisera in 190 of 192 field isolates.

Serology of nodule isolates. Serogroup II was dominant in nodules from both topographic positions, particularly the bottomland position (Fig. 1). Serogroup III isolates appeared almost three times more frequently on the south slope than on the bottomland. Serogroup I isolates were also more frequent on the south slope. Serogroup II comprised 52% of

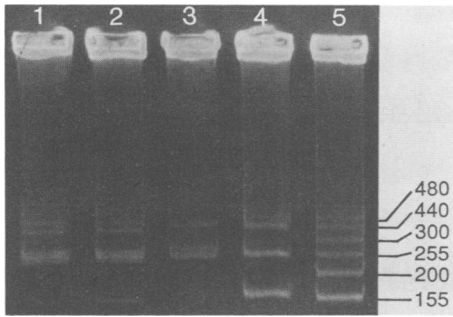


FIG. 2. Plasmid profiles of plasmid groups 5, 8, 15, and 18 (lanes 1 to 4, respectively) and kilobase size (numbers at right) markers of *R. leguminosarum* T83K3 (lane 5) in a 0.9% agarose gel.

the isolates, and the remaining isolates were split approximately equally between serogroups I and III. The three serogroups comprised 93% of the root nodule isolates, with 6% not reacting and 1% cross-reacting.

Plasmid profiles. Profiles consisted of from two to six plasmids ranging from approximately 155 to 450 kilobases (Fig. 2). The plasmid profiles were reproducible, although an indistinct high-molecular-weight band (considerably larger than a 500-kilobase plasmid) was commonly present or absent in all lanes of a particular gel and was therefore considered an artifact. Eighteen plasmid groups were identified (data not shown); 8 groups contained 10 or more isolates. Fifteen isolates (8%) had unique plasmid profiles (data not shown). Seventy-eight percent of the isolates within plasmid groups were present in the eight major plasmid groups. Thirteen of the plasmid groups were found to differ from another plasmid group by the addition of a single plasmid or the presence of a single plasmid of a different size. In addition, 7 of the 15 plasmid profiles containing only one isolate differed from a plasmid group identity by only one plasmid.

Individual plasmid groups were specific for a particular serogroup, e.g., all 22 isolates of plasmid group 3 were of serogroup II (Fig. 3). Isolates within plasmid groups were specific for a serogroup in 94% of the study population. Two plasmid groups dominated each serogroup (Fig. 3). Most plasmid groups showed a preference for a particular topographic position, most notably the exclusive occurrence of plasmid group 11 on the south slope and the nearly exclusive occurrence of plasmid group 3 on the bottomland (Fig. 3). Isolates within plasmid groups were specific for a topographic position in 76% of the study population.

IAR. Eight IAR clusters were identified, with Euclidean distances for individual clusters ranging from 1.7 to 2.5 (data not shown). Individual clusters were defined by one to three highly significant antibiotics (Table 1), with kanamycin, streptomycin, novobiocin, and tetracycline of primary importance in collectively defining the eight clusters (data not shown). IAR clusters were associated with specific serogroups (Fig. 4). Resistance to erythromycin was associated exclusively with serogroup I, resistance to kanamycin, sensitivity to vancomycin, and resistance to neomycin was associated exclusively with serogroup III, and sensitivity to erythromycin, kanamycin, and neomycin characterized serogroup II (Table 1 and Fig. 4). Reaction to tetracycline was central in separating serogroup III into IAR clusters 2 and 3, and reactions to novobiocin, streptomycin, and carbenicillin were central in separating serogroup II into IAR clusters 4 through 7. Isolates within each IAR cluster were specific for

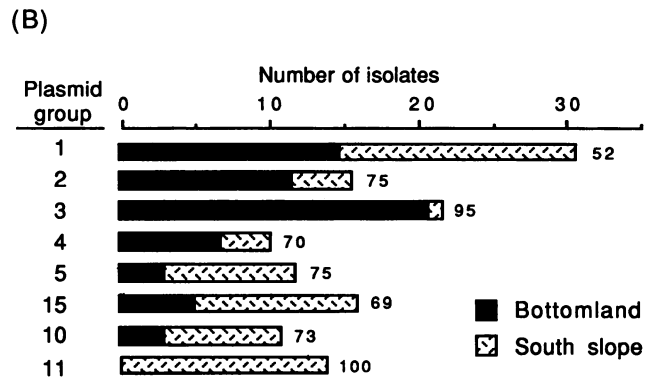


FIG. 3. Distribution of isolates within each plasmid group by their serogroup identity (A) and position of isolation (B). Numbers to the right of bars show the percentages of isolates in the plasmid group that occurred in the dominant serogroup of the plasmid group (A) or in the dominant topographic position of the plasmid group (B). Plasmid groups containing 10 or more isolates are shown.

a serogroup in 84% of the study population. IAR clusters 1, 2, and 5 were associated with a specific topographic position (Fig. 4). Isolates within each IAR cluster were specific for a topographic position in 68% of the study population. Resistance to tetracycline, erythromycin, kanamycin, and neomycin were 5.6, 3.8, 3.5, and 3.4 times more frequent, respectively, among south slope isolates than among bottomland isolates.

TABLE 1. IAR cluster definitions

IAR cluster	No. of isolates	Antibiotics defining the cluster ^a
1	23	ERY^r CHL^s CAR^s NEO^s STR^s TET^s kan^s nov^s
2	22	KAN^r TET^r VAN^s NOV^s STR^s neo^r rif^r
3	13	KAN^r NEO^r VAN^s TET^s nov^s str^s
4	25	NOV^r KAN^s TET^s car^r ery^s neo^s
5	31	STR^r ERY^s KAN^s NEO^s NOV^s TET^s
6	26	CAR^r ERY^s KAN^s NOV^s STR^s TET^s neo^s rif^s
7	41	CAR^s ERY^s KAN^s STR^s TET^s chl^s neo^s nov^s rif^s
8	11	TET^r CHL^s KAN^s NOV^s STR^s rif^s

^a Antibiotic reaction is listed in order of decreasing significance. Uppercase letters show antibiotics for which 100% of the cluster members were either resistant (r) or sensitive (s). Lowercase letters show antibiotics for which greater than 90% of the cluster members were either resistant or sensitive. Boldface type signifies antibiotics which highly defined the cluster (data not shown).

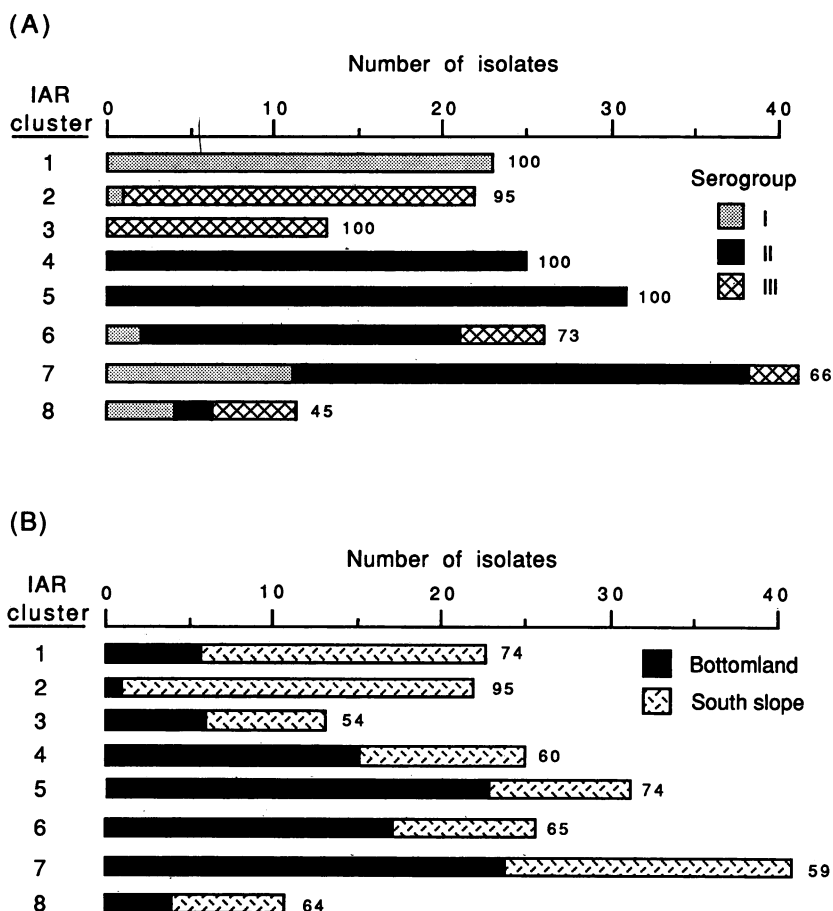


FIG. 4. Distribution of isolates within each IAR cluster by their serogroup identity (A) and position of isolation (B). Numbers to the right of bars show the percentages of isolates in the IAR cluster that occurred in the dominant serogroup of the IAR cluster (A) or in the dominant topographic position of the IAR cluster (B).

IAR clusters were also often associated with specific plasmid groups (Fig. 5). For example, plasmid group 11 dominated IAR cluster 2, with 86% of all plasmid group 11 isolates occurring within the IAR cluster. A total of 72% of the study population was grouped similarly by the plasmid profile and IAR methodologies.

DISCUSSION

Many studies with nodule isolates have illustrated that natural rhizobial populations, and specifically serogroups, comprised a multitude of strains. Heterogeneity in plasmid profiles (12, 21, 42, 51), IAR (2, 21, 33), symbiotic plasmid restriction polymorphism (57), enzyme polymorphism types (55-57), and bacteriophage typing (8, 35) of nodule isolates has been demonstrated. However, serology was not investigated in these studies. Within serogroups, variability is present in plasmid profile analysis (23), symbiotic plasmid restriction polymorphism (50), IAR, (3, 31, 52), protein profiles (16, 27, 30, 43), bacteriophage typing (32, 43), and symbiotic effectiveness (16, 20, 50, 52). Often a minority of strains dominate a serogroup, whereas a large number of strains in the same serogroup appear infrequently in nodules (16, 23, 27, 31, 43, 52), suggesting that qualitative and/or quantitative differences exist in the composition of the soil population.

Thus, the diversity found in the 192 pea nodule isolates from two topographic positions in eastern Washington was not unexpected. A total of 33 different plasmid profiles were observed, with 19 of these differing by only one plasmid from another plasmid profile. Eight IAR clusters containing isolates with identical or very similar reactions were identified. Serogroup II isolates possessed the greatest variability in plasmid profiles and response to antibiotics.

A surprising finding of this study was the number of strong associations observed within the diversity. Ninety-three percent of the study population consisted of three serogroups, allowing an in-depth examination of serogroup-plasmid group and serogroup-IAR relationships. Isolates within plasmid groups and IAR clusters were highly specific (94 and 84%, respectively) for a serogroup. In addition, each serogroup was dominated by two plasmid groups in nodules. There was also a large overlap between groupings determined by plasmid content and IAR. Thus, the three properties are strongly associated in nodule isolates from field-grown peas at the study site in the Palouse region of eastern Washington.

Other investigators have found single associations when using multiple methodologies for studying nodule isolates. These include associations between a dominant IAR and serology (31), a dominant plasmid profile and IAR (39),

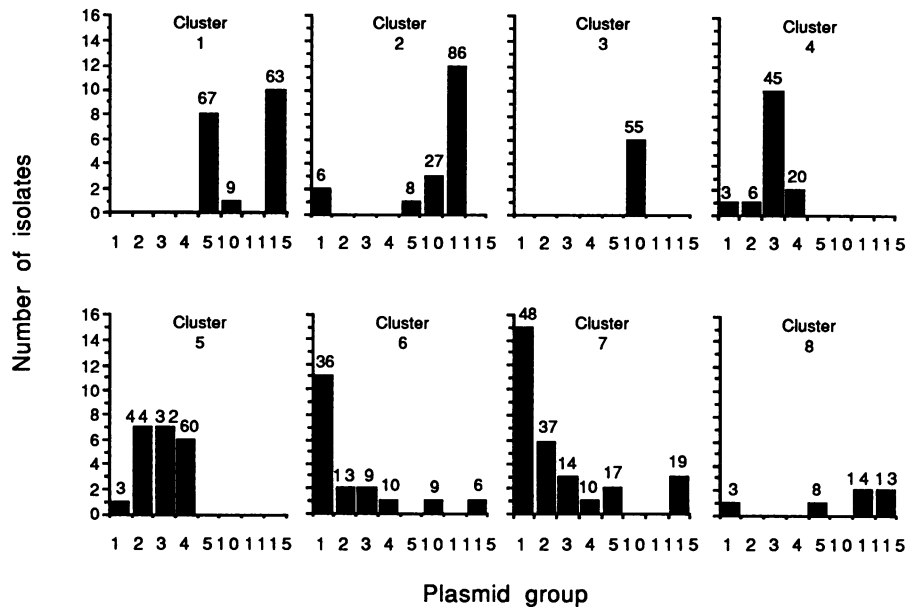


FIG. 5. Distribution of isolates within each IAR cluster by their plasmid group identity. Numbers above bars show the percentages of isolates in a plasmid group represented in each IAR cluster. Plasmid groups containing 10 or more isolates are shown.

protein profiles and serology (11, 27, 30, 43), protein profiles and enzyme polymorphism types (55), and symbiotic plasmid restriction polymorphism and enzyme polymorphism types (57). The occurrence of dominant plasmid groups in nodules has also been noted (23, 39, 42). Prior to this study, only Broughton et al. (9) had simultaneously studied serology, plasmid profiles, and IAR. They found no associations among these properties. The lack of association may have been due to the fact that nodule isolates were from a soil planted only once with the host legume. In the Palouse, 80 years of intensive pea cultivation (41) may have provided continuous selection for adapted genotypes, resulting in the strong associations among serology, plasmid profiles, and IAR.

Most plasmid profiles showed more resemblance to other plasmid profiles or groups within the serogroup than to plasmid profiles or groups within other serogroups. However, there were five instances of a plasmid of the same apparent mobility being distributed within two serogroups (data not shown). Thus, the possibility exists that plasmid transfer between strains of different serogroups may occur in the Palouse field environment. Clearly, plasmid restriction studies are necessary to determine to what extent plasmids of apparent identical mobility are structurally identical. Two recent reports (50, 57) have provided evidence that plasmid transfer among rhizobia must occur in the field.

Topographic position was only weakly associated with serogroup distribution in nodules. Serogroup distribution on the south slope and bottomland contrasted with that found in previous studies (37, 38, 52). Furthermore, serogroup III was more prevalent in both topographic positions in this study. One explanation is that the host plant cultivar used in this study was Latah, while Alaska was used in previous studies. Host plant cultivar effects on serogroup recovery (13, 49, 52) and on enzyme polymorphism types (55) in nodules has been demonstrated.

To our knowledge this is the first topographic study of intraserogroup nodule isolates. Isolates within plasmid groups and IAR clusters were somewhat specific (76 and

68%, respectively) for a topographic position, with several plasmid groups and IAR clusters being highly specific for a topographic position. This result suggests that the genetic composition of the population varies considerably between locations only 110 m apart. Topographically determined differences in soil microclimate may favor the growth, survival, or competitiveness for nodulation of specific intraserogroup strains.

The effect that topographically determined differences in soil microclimate may have in producing different microbial communities must also be considered. Soil temperature (40; K. A. Kauffmann, M.S. thesis, Washington State University, Pullman, 1987) and moisture (34, 45) may vary with topographic position and affect the populations, activities, or metabolites of other soil inhabitants. These effects could then limit the relative abundance and/or diversity of rhizobial strains available for selection by the host plant. For example, the prevalence of low-level antibiotic resistance in nodule isolates from the bottomland topographic position (versus the south slope) could have resulted from antibiotic-producing antagonists that preferentially inhabited the wetter, cooler bottomland soils.

The recognition of intraserogroup strains is important in rhizobial ecology for several reasons. As shown in this study, specific plasmid groups within a serogroup are predominant in different topographic positions. Studies using serology alone (19) may fail to observe the effect of topographic position on the recovery of specific strains from nodules. In addition, the use of serology alone fails to address whether the host plant can preferentially select certain strains from a diverse serogroup population. Finally, laboratory and greenhouse studies with a single isolate of a serogroup(s) are not representative of the diverse serogroup population present in the field.

The usefulness of plasmid profile analysis at other locations will need to be tested on a case-by-case basis because the frequencies of plasmid transfer (50; Young and Wexler, in press), transduction (11), conjugation (4, 7, 10, 15, 28), plasmid recombination and deletion (4, 6, 26, 29), and other

genetic changes will alter a particular pattern. In addition, strain-specific DNA probes may be of use in identifying intraserogroup strains with high competitiveness for nodule occupancy. A more detailed sampling of spatial variability between nodules on a plant, plants across the topography, and soil across the topography should also be addressed in future studies.

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