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Strain identification in *Rhizobium meliloti* using the antibiotic disk susceptibility test*

H. ANTOUN,
Département des Sols, Faculté des sciences de l'Agriculture et de l'Alimentation, Université Laval, Québec, Canada, GIK 7P4

L. M. BORDELEAU and DANIELLE PREVOST
Station de Recherches, Agriculture Canada, 2560, Boulevard Hochelaga, Sainte-Foy Québec, Canada, GIV 2J3

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Summary The antibiotic disk susceptibility test was used to measure the variation in the intrinsic resistance of 49 strains of *Rhizobium meliloti* to 9 antibiotics. Several strains had unique patterns of resistance. However, during cluster analysis, when a minimum Euclidean distance equal to 4 was used as a discriminating tool, the strains were grouped in 12 groups. The largest group contained 74% of the strains but 9 strains (2 very effective, 4 effective and 3 ineffective) showed very unique patterns of resistance and formed 9 distinct groups. *R. meliloti* strains in general showed high intrinsic resistance to the 9 antibiotics tested.

Introduction

One of the major problems encountered during the study of *Rhizobium meliloti* ecology is the strain identification. The use of mutants in ecological study is not reliable because genetically marked strains with high levels of antibiotic resistance or auxotrophy can be affected in their symbiotic properties[4,7,10]. Serological techniques have severe limitations when applied to *R. meliloti*[8] and showed that the strains can only be separated into different groups, but identification of strains within a group is not feasible[12]. The use of two-dimensional polyacrylamide gel electrophoresis showed that among other *Rhizobium* species, *R. meliloti* formed a distinct group and that this technique can be used to show differences between similar strains[11]. However, before an identification based on gels can be considered to be reliable, many more *R. meliloti* strains must be tested. The intrinsic antibiotic resistance have been used to identify *R. leguminosarum* and *R. phaseoli* strains under laboratory conditions[9], and to identify inoculum strains of *R. phaseoli* under field conditions[2].

The present work show that some *R. meliloti* strains can be clearly identified under laboratory conditions with the antibiotic disk susceptibility test described for clinical bacteria[5].

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Material and methods

Strains of *R. meliloti* used in this study and their symbiotic efficiency were previously described[3]. The strains were cultured on YMB liquid medium[14] containing (g/l): mannitol 10; Difco Bacto yeast Extract 1; K_2HPO_4 0.5; $MgSO_4.7H_2O$ 0.2; NaCl 0.1; pH 7.0. The strains were incubated for 4 days at 20°C on a rotary shaker and suspended in sterile saline to give approximately 10^7 cells/ml. Dried plates, containing YMB medium solidified with 15 g/l of Difco Bacto agar, were surface inoculated with the diluted cultures, with a sterile cotton swab[5]. In alternate trials, the strains were cultured on slants of the solidified YMB medium and suspended directly in sterile saline without any change in the antibiotic resistance pattern. Difco Bacto antibiotic disks were applied on the plates with a dispenser, and after 3–4 days incubation at 28°C the strains were considered to be resistant, intermediate or sensitive to the antibiotics tested according to the diameters of the inhibition zones as described for clinical bacteria[5]. The antibiotic disks used were the following: aureomycin 5, 10 and 30 μg (A_5, A_{10}, A_{30}); bacitracin 2, 5 and 10 units (B_2, B_5, B_{10}); carbenicillin 50 and 100 μg (CB_{50}, CB_{100}); chloramphenicol 5, 10 and 30 μg (C_5, C_{10}, C_{30}); kanamycin 5, 10 and 30 μg (K_5, K_{10}, K_{30}); nalidixic acid 10 and 30 μg (NA_{10}, NA_{30}); neomycin 5, 10 and 30 μg (N_5, N_{10}, N_{30}); novobiocin 5, 10 and 30 μg (NB_5, NB_{10}, NB_{30}); polymixin B 50, 100, 300 units (PB_{50}, PB_{100}, PB_{300}); streptomycin 2, 5 and 10 μg (S_2, S_5, S_{10}); oxytetracycline 10 and 30 μg (O_{10}, O_{30}); tetracycline 5, 10 and 30 μg (T_5, T_{10}, T_{30}); triple sulfa 50 and 150 μg (TS_{50}, TS_{150}).

Cluster analysis was carried out by using BMD-P2M computer program[6]. Results were recorded as follows: strain resistant to the antibiotic tested = 3, intermediate resistance = 2 and sensitive = 1. The Euclidean distance between cases and clusters was calculated. The results are shown as a dendogram prepared by the weighed average linkage method[13].

Results and discussion

All the *R. meliloti* strains tested were sensitive to aureomycin (A_5, A_{10}, A_{30}), oxytetracycline (O_{10}, O_{30}), tetracycline (T_5, T_{10}, T_{30}) and resistant to bacitracin (B_2, B_5), kanamycin (K_5), neomycin (N_5, N_{10}, N_{30}) and polymixin B (PB_{50}, PB_{100}). These antibiotic disks were not used for further identification studies of the *R. meliloti* strains. The resistance patterns of the 49 strains tested is shown in Table 1. Very little variability was observed with some strains rated intermediate in resistance mainly when the diameter of the inhibition zone was very close to the limits established between two classes of resistance[5]. Several strains showed unique pattern of resistance, however in studies directed toward the re-isolation of an introduced strain from soils containing indigenous population, a severe discrimination method have to be used to avoid the effect of any variability that could occur. The use of the Euclidean distance in taxonomy has the disadvantage of grouping some slightly dissimilar strains in the same cluster, as for strains A_4 and S_{15}, or in different clusters as for strains S_4 and S_8 (Fig. 1 and Table 1). However, if a high Euclidean distance is established as a base for discriminating between two clusters, this disadvantage will be overcomed and we will have a more strict tool to characterize the different strains. By considering a value of 4 as the minimum acceptable Euclidean distance to differentiate between 2 clusters, the 49 strains of *R. meliloti* will form 12 distinct groups (Fig. 1 and Table 1).

Table 2. Effect of the antibiotic disks tested on the 49 strains of *R. meliloti*

Antibiotic disks	Number of strains		
	Resistant	Intermediate	Sensitive
B_{10}	46	2	1
CB_{50}	42	0	7
CB_{100}	42	0	7
C_5	48	0	1
C_{10}	45	2	2
C_{30}	42	1	6
K_{10}	47	2	0
K_{30}	41	4	4
NA_{10}	46	0	3
NA_{30}	40	4	5
NB_5	48	0	1
NB_{10}	45	1	3
NB_{30}	31	7	11
PB_{300}	34	14	1
S_2	5	31	13
S_5	4	10	35
S_{10}	2	1	46
TS_{50}	46	0	3
TS_{150}	46	0	3
Total (931)	700	79	152
%	75.2	8.5	16.3

Group 9 is the largest group and it contains 15 clusters formed by approximately 74% of the strains, but most of the remaining 26% of the strains can be identified without any ambiguity. In fact, the very effective strains S_{14} and 3Doa8, the effective strains S_9, S_{22}, V_4 and 54033 and the ineffective strains E_1, A_1 and C_1 form 9 distinct groups showing unique pattern of resistance and could be used efficiently in ecological studies. The highest Euclidean distance observed with the ineffective strains C_1 and A_1, might indicate that these strains belong to other *Rhizobium* species and that the ineffective strains have to be carefully studied to determine their exact identity with other techniques[11]. No correlation was observed between the effectiveness of the strains and their antibiotic resistance. These results confirm the previous observations made with *R. trifolii*[7].

R. meliloti showed high intrinsic antibiotic resistance to the 9 antibiotics tested. In fact, the strains were resistant to 75% of the total antibiotic disks tested and sensitive to only 16% of the disks (Table 2). These data could be useful in formulating media in studies where *R. meliloti* has to be isolated directly from soils[1].

Table 1. Antibiotic-resistance patterns for the *Rhizobium meliloti* strains used in this study

Group no.	Strains	Antibiotic disks*																	
		B_{10}	CB_{50}	CB_{100}	C_5	C_{10}	C_{30}	K_{10}	K_{30}	NA_{10}	NA_{30}	NB_5	NB_{10}	NB_{30}	PB_{300}	S_2	S_5	S_{10}	TS_{50}	TS_{150}
1	S_{14}	R	R	R	S	S	S	R	R	R	R	R	R	S	R	I	S	S	R	R
2	3Doa8	R	S	S	R	R	R	I	S	R	R	R	R	R	R	I	I	S	R	R
3	V_4	R	S	S	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
4	S_{22}	R	S	S	R	I	S	R	R	R	S	R	R	R	R	I	I	S	R	R
5	54033	R	R	R	R	R	R	R	R	R	R	R	R	I	I	S	S	S	S	S
6	S_9	I	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R	I	R	R
7	A_4	R	R	R	R	R	S	R	R	R	R	R	I	S	R	I	S	S	R	R
	S_{15}	I	R	R	R	R	S	R	R	R	R	R	S	S	R	I	S	S	R	R
8	E_1	R	R	R	R	R	R	R	S	R	R	R	R	S	I	S	S	S	R	R
9a	V_7	R	R	R	R	R	R	R	R	R	R	R	R	S	I	S	S	S	R	R
b	S_{11}, R_1	R	R	R	R	R	R	R	R	R	R	R	R	S	R	I	S	S	R	R
c	D_3	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S	S	R	R
d	$A_2, S_6, S_{12}, I_2, I_3, I_4$	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S	S	S	R	R
e	$S_1, S_2, S_{13}, S_{20}, V_2$	R	R	R	R	R	R	R	R	R	R	R	R	R	R	I	I	S	R	R
f	S_4	R	R	R	R	R	R	R	R	R	I	R	R	R	R	I	I	S	R	R
g	S_8	R	R	R	R	R	R	R	R	R	I	R	R	R	R	I	S	S	R	R
h	E_2	R	R	R	R	R	R	R	R	R	R	R	R	I	R	I	S	S	R	R
i	23A, A_3, S_7, S_{10}, S_{16}, S_{19}, V_5	R	R	R	R	R	R	R	R	R	R	R	R	R	R	I	S	S	R	R
j	3Doa20a	R	R	R	R	R	R	R	I	R	R	R	R	R	R	I	S	S	R	R
	S_3	R	R	R	R	R	R	R	I	R	I	R	R	R	R	I	S	S	R	R
k	S_5	R	R	R	R	R	R	R	I	R	R	R	R	R	I	S	S	S	R	R
	V_1	R	R	R	R	R	R	R	R	R	R	R	R	R	I	S	S	S	R	R
l	A_5, D_2	R	R	R	R	R	R	R	R	R	R	R	R	I	I	I	I	S	R	R
m	D_1	R	R	R	R	R	R	R	R	R	I	R	R	R	I	I	S	S	R	R
n	S_{21}, V_6	R	R	R	R	R	R	R	R	R	R	R	R	R	I	I	S	S	R	R
0	I_1, V_3	R	R	R	R	R	R	R	R	R	R	R	R	I	I	I	S	S	R	R
10	ALFALFA D	R	S	S	R	R	I	R	I	S	S	R	R	I	I	S	S	S	S	S
	54032	R	S	S	R	I	S	R	R	S	S	R	R	S	I	S	S	S	S	S
11	A_1	S	S	S	R	R	R	I	S	R	S	R	S	S	R	R	R	S	R	R
12	C_1	R	S	S	R	S	S	R	S	S	S	S	S	S	S	R	R	R	R	R

* For the description of the antibiotic disks used see Material and Methods.
R = Resistant I = Intermediate S = Sensitive.

Fig. 1. Dendogram, based on the Euclidean distance, of the *Rhizobium meliloti* strains according to their intrinsic antibiotic resistance patterns.

The method described in this work show that some strains of *R. meliloti* can be easily identified under laboratory conditions. This method can probably be improved by using other intrinsic properties such as specific requirements, carbon and nitrogen utilisation. Field trials work are required to evaluate the reliability of this method in the *in situ* ecological studies of *R. meliloti*.

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