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Adsorption and Selection of Rhizobia with Ion-Exchange Papers

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Abstract. Ion exchange papers were used to study the adsorption of ^{32}P-labelled rhizobia on defined surfaces. Two strains of *Rhizobium japonicum* and one each of *R. leguminosarum* and *R. lupini* were compared with *Escherichia coli* and *Bacillus subtilis*. The ratio of adsorption to strong and to weak acid papers/strong and weak basic papers was consistantly higher for all rhizobial strains compared to the other bacteria. The process of desorption by increasing the ion-concentration causes about 35% desorption between 0.02 and 0.1 M $MgCl_2$, however, an increase to 1 M does not desorb more labelled *Rhizobium japonicum* or *E. coli* cells. The ratio of adsorbed cpm to colony formers, desorbed by 0.1 M NaCl was similar with *Rhizobium japonicum* for all six ion exchange papers. For *E. coli* this ratio varied widely for the different papers. The selection of *Rhizobium* against a more closely related bacterium by this adsorption/desorption procedure was demonstrated with mixed cultures of *Rhizobium japonicum* and *Chromobacterium violaceum* giving a more than 80 fold enrichment of the former. *Rhizobium japonicum* cells, ad/desorbed from all ion exchange papers kept their infectivity and formed nodules on *Glycine max* with an activity of 20–40 nM $C_2H_4 \cdot hr^{-1} \cdot mg\ nodule^{-1}$. A desorption of *Rhizobium japonicum* from soybean roots also occurred by increasing the ion concentration. 2–3 times as many cells were removed in this way compared to washing with water.

Key words: Rhizobium japonicum – *Rhizobium leguminosarum* – *Rhizobium lupini* – Adhesion – Ion Exchange-Papers – Selection Method.

The cellular and molecular basis of specificity of rhizobia for the infection of legumes is still unknown. The systematics of rhizobia still depend almost completely on the infection range of the bacteria (Bergeys Manual, 1974). The first infection of a non-legume, *Trema cannabina* (Ulmaceae) by a cowpea *Rhizobium* strain (Trinick, 1973) and the much less specific behaviour of rhizobial strains in association with tissue cultures compared to infection of whole plants (Child, 1975; Snowcroft and Gibson, 1975) stimulated studies concerning these questions. The separation of the first "recognizing step" (Dart, 1974) from the later events in the symbiotic development is a difficult methodogical problem. Analytical studies of the extracellular carbohydrate, nucleic acid and protein composition of four *Rhizobium japonicum* strains, differing considerably in their nodulating ability, gave no significant differences (Keele *et al.*, 1974). But 60–80% of the excreted material in this study could not be identified. Also the sugar composition, in particular the absence of mannose and galactose in the extracellular polysaccharide of slow growing and of fast growing rhizobia, is still questioned (Humphrey *et al.*, 1974). Serological studies have revealed, that within the same serogroup of *Rhizobium japonicum* considerable variation towards inhibition of nodulation by nitrate and tolerance of fungicides existed (Gibson *et al.*, 1971). Analytical and serological experiments can be supported by direct studies of the first steps of rhizobia-plant cell interaction. At this stage, we preferred to use instead of plant cells, ion exchange papers as defined surfaces (Dorfner, 1964) in the study of adsorptive properties of rhizobia. The present work was intended to evaluate ad- and desorption qualities of different rhizobial strains and to compare these qualities for other bacterial species. We wanted also to develop this method into a special selection procedure for rhizobia versus other soil bacteria like *Chromobacterium violaceum*.

Abbreviations Used. IEP = ion-exchange papers; CM-C = Carboxymethyl-cellulose; DEAE-C = DEAE-cellulose.

Materials and Methods

Bacterial Strains and Culture Conditions. Rhizobium japonicum strain 85 was received from the USDA, Beltsville, Md., by courtesy of Dr. Weber, *Rhizobium japonicum* strain 61-A-96 from the Nitragin Company, Milwaukee, by courtesy of Dr. Burton, *Rhizobium leguminosarum* strain C 53 from Dr. H. J. Evans, Oregon State University, Corvallis, Oregon, and *Rhizobium lupini* strain 7-1 from Dr. Heumann, University of Erlangen. *Escherichia coli K 12* ATCC, *Chromobacterium violaceum* strain CCM 2076 and *Bacillus subtilis* were supplied by Dr. Mannheim of this university. All strains were routinely cultured in a new low phosphate and low nitrogen medium especially developed for rhizobia (No. 20 E) with the following

composition: 1000 ml medium contained 68 mg KH_2PO_4, 87 mg K_2HPO_4, 370 mg $MgSO_4 \times 7H_2O$, 73.5 mg $CaCl_2 \times 2H_2O$, 506 mg KNO_3, 4.84 mg $Na_2MoO_4 \times 2H_2O$, 6.95 mg $FeSO_4 \times 7H_2O$ as an EDTA-complex with 9.3 mg EDTA, 4.6 g glycerol, 1.82 g mannitol and 2.09 yeast extract (Merck), the pH was 6.8. The suspensions were grown at 28°C on a gyrorotary shaker (120 rpm) in batch cultures with 100 ml medium.

Labelling of the Bacteria with $^{32}PO_4$ and Cerenkov-Counting. To increase the specific activity of the bacteria, cells from logarithmically growing cultures were centrifuged, washed and resuspended in a phosphate free medium with 0.02 M TES-Puffer pH 6.8 and with only 10% of the regular yeast extract concentration. 20 µCi $^{32}PO_4$ (received weekly from Amersham-Buchler, Frankfurt) were added. After 24 hrs of growth, the bacteria were harvested by centrifugation (10 min at 38000 × g) at 20°C, washed twice with and resuspended in distilled water. The radioactivity was determined by Cerenkov counting in a Packard model 3380 with quench correction by applying channel ratio method. The efficiency was between 40 and 20%. The specific activity of the bacteria was determined between 1000 and 1500 cells/cpm.

Adsorption and Desorption Experiments. 5 ml bacterial suspension were incubated for 15 min with 2 ion-exchange papers (1.5 × 4 cm) in small Petri dishes on a rotary shaker with slow movement (30 rpm). The ion exchange papers (SB2, SA 2, WB 2, WA 2, Carboxymethyl-cellulose and DEAE-cellulose) were purchased from Serva, Heidelberg. The papers were washed thoroughly 3 times in 0.5 l distilled water. For measurement of the adsorbed activity, the washed papers were put in LSC vessels and counted after addition of 10 ml of water. For the desorption experiments, the papers were incubated in 10 ml of NaCl or $MgCl_2$ solution from 0.02 to 1.0 M for 5 min. The suspension of desorbed cells was either counted for radioactivity or – after appropriate dilution – aliquots were plated on agar solidified medium 20E for assessment of colony formers.

Infectivity Testing with Glycine max. Seeds of *Glycine max* var. Chippeway were surface sterilized by using UV-light and a 2% solution of $CaOCl_2$ and germinated for 14 days in perlite (Perlite-Dämmstoff GmbH, Dortmund). Bacterial colonies, developed from single desorbed rhizobial cells were suspended in 5 ml water with 0.15% agar (Merck, purified) and the root system of the seedling allowed to stand for 5 min in the suspension. The infected seedling was planted in a new pot with perlite incubated in a phytotron with a 14 – 10 hr light-dark regime, 25°C and 75% humidity during the light phase (18000 cd/s × m²) and 20°C and 75% humidity at night. The plants were watered every second day with the following nitrogen free legume-nutrient solution (sterilized): 1000 ml medium contained 279 mg K_2SO_4, 493 mg $MgSO_4 \times 7H_2O$, 23 mg KH_2PO_4, 145 mg K_2HPO_4, 371 mg $CaCl_2 \times 2H_2O$, 1.43 mg H_3BO_3, 1.02 mg $MnSO_4 \times 4H_2O$, 0.22 mg $ZnSO_4 \times 7H_2O$, 0.08 mg $CuSO_4 \times 5H_2O$, 0.05 mg $Na_2MoO_4 \times 4H_2O$, 0.10 mg $CoCl_2 \times 4H_2O$, 16.7 mg Fe-EDTA-di-o-hydroxyphenylacetate (Geigy Chemicals, Yonkers, N.Y.). The pH was 7.0. Once a week, the plants were watered with sterilized tap-water. Four weeks after infection, the whole root system of the plants was cut off, incubated for 1 hr in reagent tubes (volume 60 ml) and tested for acetylene reducing activity as described previously (Wilcockson *et al.*, in preparation). Finally the nodules were picked from the plants and the fresh weight determined. For adsorption experiments with the root system of *Glycine max* 17 days old plants, germinated in perlite and cultured under the same conditions as above, were used.

Table 1. Adsorption of ^{32}P-labelled bacteria on ion-exchange papers. Adsorbed cpm from 5 ml bacterial suspension with 5×10^5 cpm. Specific activity: 1000 – 1500 cells/cpm. Ratio of No.: in brackets average of 4 parallels

Ion exchange No., type and ratio of No.	cpm from *Rhizobium japonicum* st. 85	cpm from *Rhizobium japonicum* st. 61-A-96	cpm from *Rhizobium leguminosarum* st. C 53
1: SB 2	140882	26770	62280
2: SA 2	32558	9480	20803
3: WB 2	44160	15876	39268
4: WA 2	28771	8316	18498
5: CM-C	13260	3037	9966
6: DEAE-C	104095	58036	106410
2/1	0.23 (0.24)	0.35 (0.40)	0.33 (0.35)
4/3	0.65 (0.62)	0.52 (0.53)	0.47 (0.43)
4/6	0.27 (0.25)	0.14 (0.17)	0.17 (0.14)
5/6	0.13 (0.09)	0.05 (0.08)	0.09 (0.09)
Ion exchange No., type and ratio of No.	cpm from *Rhizobium lupini* st. 7-1	cpm from *Escherichia coli* K 12 ATCC	cpm from *Bacillus subtilis*
1: SB 2	45944	40299	105719
2: SA 2	20463	4830	15449
3: WB 2	11047	15208	36480
4: WA 2	23822	3149	11828
5: CM-C	11700	1362	5472
6: DEAE-C	67878	35905	173799
2/1	0.44 (0.62)	0.12 (0.13)	0.15 (0.13)
4/3	2.16 (1.67)	0.21 (0.17)	0.32 (–)
4/6	0.35 (0.28)	0.09 (0.08)	0.07 (0.10)
5/6	0.17 (0.15)	0.038 (0.038)	0.03 (0.025)

Results

Data for the adsorption of ^{32}P-labelled bacteria to the six different ion-exchange papers are summarized in Table 1. Especially with the strong basic type SB 2 and the weak basic DEAE-cellulose papers, a quite high percentage (up to 30 – 35%) are adsorbed to the papers[1]. With all bacteria used least are adsorbed to the weak acid carboxymethyl-cellulose paper. Differences between the bacterial strains can be illustrated well by comparing their adsorption on two papers. The ratios, paper 2/1 and also 4/3 are significantly higher for all four strains of *Rhizobium* than for *Escherichia coli* or *Bacillus subtilis*. This means that relatively more cells of *Rhizobium* species are adsorbed to strong and to weak acid surfaces compared to strong and weak basic surfaces than for other bacteria. Similarly the ratio 5/6 (weak acid CM-C/weak basic DEAE-C) is again higher for all four strains of *Rhizobium* compared to the other strains. By the same

[1] These bacteria cannot be washed off with distilled water.

ratio method, *Rhizobium lupini* (strain 7-1) can be distinguished from the other rhizobial strains. All four ratios formed in Table 1 are significantly higher for *Rhizobium lupini* (strain 7-1). This is the only strain with which absolutely more cells are adsorbed to the weak acid paper (No. 4) than to the weak basic paper (No. 3). To the many other differences, we have noticed between the two strains of *Rhizobium japonicum* (85 and 61-A-96: growth rate, formation of extracellular components, infectivity with *Glycine* plants and *Glycine* tissue cultures), we must now add the absolute adsorption to ion exchange papers.

The specific and significant adsorption of cells of *Rhizobium* raised questions concerning the ionic conditions resulting in desorption of cells. Fig. 1 illustrates the course of desorption from the strong basic paper SB 2 by raising the molarity from zero to 0.02 M and then further up to 1.0 M using $MgCl_2$ and NaCl. The first two points indicate, that only a very small portion of the adsorbed cells are detached by a further incubation in distilled water. Almost 40% of the cells, that are detached at all, are desorbed by 0.02 M $MgCl_2$ and by 0.04 M NaCl. At 0.10 M $MgCl_2$ or 0.20 M NaCl the desorption process is almost complete and a further increase to a 1 M results in only very little further detachment. Of all the radioactivity adsorbed to the paper, about 60% remained after the desorption procedure described. The desorption from DEAE-cellulose paper (Fig. 2) the type with the second highest adsorption, shows a similar course, using NaCl as desorbent. A major proportion of the bacteria is desorbed between 0.02 and 0.06 M NaCl and only a minor fraction by a further increase to 0.3 M.

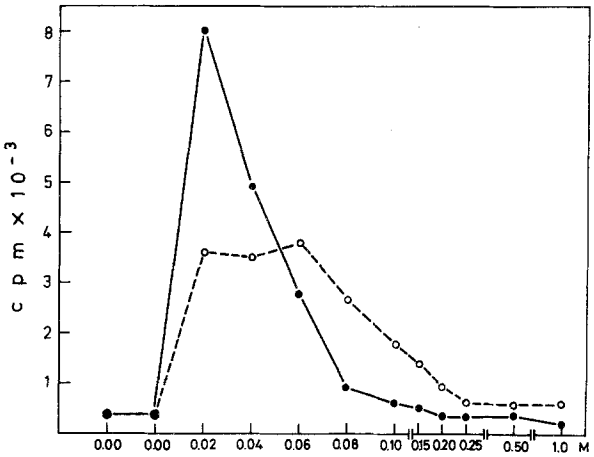
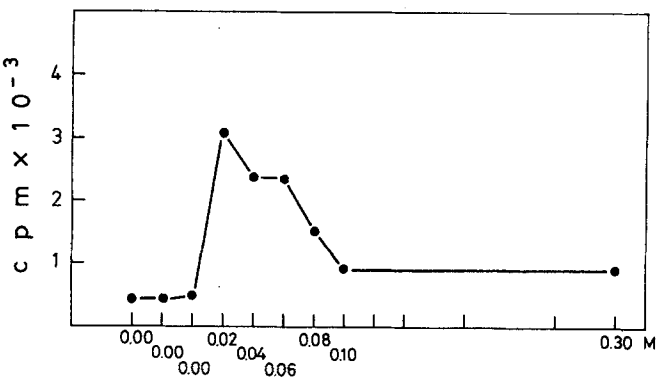


Fig. 1. Desorption of ^{32}P-labelled *Rhizobium japonicum* st. 85 from ion exchange paper SB 2. Use of increasing concentration of $MgCl_2$ (●) and NaCl (○). cpm (Cerenkov counting) desorbed from 2 papers (1.5 × 4 cm each) in 20 ml desorbing solution

A comparison with ^{32}P-labelled *E. coli* (Fig. 3) using SB 2 papers indicates that a slightly higher ionic strength is necessary to desorb the major portion of the bacteria. The sum of the number of bacteria desorbed with 0.04 and 0.06 M $MgCl_2$ is approximately the same as the number desorbed by 0.02 M $MgCl_2$ with *Rhizobium japonicum*, but about 60% higher with *E. coli*. Also with *E. coli*, between 60 and 70% of the total activity adsorbed remains bound to the SB 2 papers after the experiment illustrated in Fig. 3.

There are differences in the viability of the desorbed cells for *Rhizobium japonicum* and for *E. coli* by a factor of more than 200 using the ion-exchange papers (Table 2). The 4 ratios (Table 2) of desorbed colony forming cells from the six types of papers in *Rhizobium japonicum* st. 85 are very similar to the



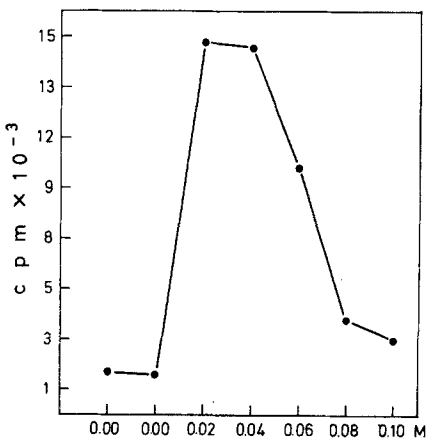


Fig. 2. Desorption of ^{32}P-labelled *Rhizobium japonicum* st. 85 from DEAE-cellulose papers. cpm desorbed by increasing concentration of NaCl from 2 papers (1.5 × 4 cm each) in 20 ml desorbing solution

Fig. 3. Desorption of ^{32}P-labelled *Escherichia coli* K 12 ATCC from ion exchange papers SB2. cpm desorbed by increasing concentrations of $MgCl_2$ from 2 papers (1.5 × 4 cm each) in 20 ml desorbing solution

4 ratios of adsorbed cpm (Table 1). However, with *E. coli*, the ratio 3/6 for the desorbed colony forming cells is 95 but the same ratio for adsorbed cpm is only 0.42. Thus in a comparison of the desorbed colony forming cells of these two bacterial strains with the DEAE-cellulose papers, there are 145 times more viable cells of *Rhizobium japonicum* st. 85 than of *E. coli*. There are only half as many with the weak basic paper WB 2.

The infectivity of the adsorbed and then desorbed cells of *Rhizobium japonicum* st. 85 was tested with plants of *Glycine max* var. Chippeway under controlled conditions in a phytotron (Table 3). The number of nodules/plant is between 23 and 32, the mg of nodules/plant (fresh weight) between 250 and 364 and the activity in nmoles $C_2H_4 \cdot hr^{-1} \cdot plant^{-1}$ between 8550 and 10125. Rhizobia which have been through the ad/desorption process keep their infectivity regardless of the type of ion-exchange paper. In another series of experiments we confirmed this also with cells, ad/desorbed from paper type WA 2, which was omitted in the series of experiments presented in Table 3. The data verify also the familiar effect, that a lower number of nodules/plant is compensated to an extent by a higher activity of the nodules. Thus the nitrogenase activity per plant has a significantly smaller variation than the number of nodules/plant.

Table 2. Estimation of ad- and desorption of bacteria with ion-exchange papers by colony counting

Ion exchange No., type and ratio of No.	*E. coli* K 12[a] Bacteria ad/desorbed from 24 cm^2 IEP	*R. japonicum* 85[b] Bacteria ad/desorbed from 24 cm^2 IEP
1: SB 2	3.2×10^6	36.8×10^6
2: SA 2	3.6×10^6	16.4×10^6
3: WB 2	38×10^6	20.4×10^6
4: WA 2	2.8×10^6	17.2×10^6
5: CM-C	3.2×10^6	6.4×10^6
6: DEAE-C	0.4×10^6	58×10^6
2/1	1.13	0.44
4/3	0.07	0.84
4/6	7.0	0.30
3/6	95	0.35

[a] Incubation in 5 ml suspension of *E. coli* with 1.5×10^9 cell/ml (colony plating).
[b] Incubation in 5 ml suspension of *R. japonicum* with 5.2×10^9 cells/ml (colony plating).

The results in Table 2 indicate a relative enrichment of *Rhizobium* against *E. coli* in separated cultures by using the ion-exchange papers. As the next step the method was further developed by using mixed cultures. To demonstrate a true selection of *Rhizobium* against other soil bacteria we choose a strain of *Chromobacterium violaceum*. Discrimination between the two bacteria is facilitated by the blue violet coloured colonies of *Chromobacterium violaceum*. In the experiment presented in Table 4, 3.1×10^9 viable cells of *Rhizobium japonicum* st. 61-A-96 and 3.6×10^{11} viable cells of *Chromobacterium violaceum* were mixed and the normal ad/desorption procedure carried out. For all six types of ion exchange papers we find an enrichment for *Rhizobia*. We obtained the most impressive selection of *Rhizobium* with type WA 2, the ratio *Rhizobium*/*Chromobacterium* was increased from 0.0086 to 0.720. A much higher concentration of bacteria (3.631×10^{11} together) was used in the experiment to guarantee a high competition for the adsorbing spots. The percentage of ad/desorbed bacteria (*Rhizobium* and *Chromobacterium* together) was only about 0.1% and much smaller than in the experiments presented in Table 2. A demonstration of enrichment for *Rhizobium japonicum* st. 61-A-96 versus *Chromobacterium violaceum* in a similar experiment is given in Fig. 4a and b. From a ratio *Rhizobium*/*Chromobacterium* of 0.02 in the original mixture we get a ratio of about 0.05 with the DEAE-cellulose type and a maximum ratio of 6.0 with type WA 2.

Experiments with the root system of *Glycine max* as an adsorbtive surface are summarized in Table 5. After incubation of the root system of 17 day-old

Table 3. Infectivity of *Rhizobium japonicum* st. 85 after ad- and desorption from ion-exchange papers with *Glycine max* var. Chippeway. Data are averages of 4 plants, harvested 4 weeks after infection

Ion exchange type	Number of nodules/plant	mg nodules (fresh weight/pl.)	Activity nmole $C_2H_4 \cdot hr^{-1} \cdot plant^{-1}$	Activity nmoles $C_2H_4 \cdot hr^{-1} \cdot mg\ nodules^{-1}$
SB 2	27	364	8550	23
SA 2	23	250	10125	40
WB 2	25	273	9000	33
CMC	27	272	8494	32
DEAE	32	318	9225	30
Control (plants without infection)	0	0	0	0

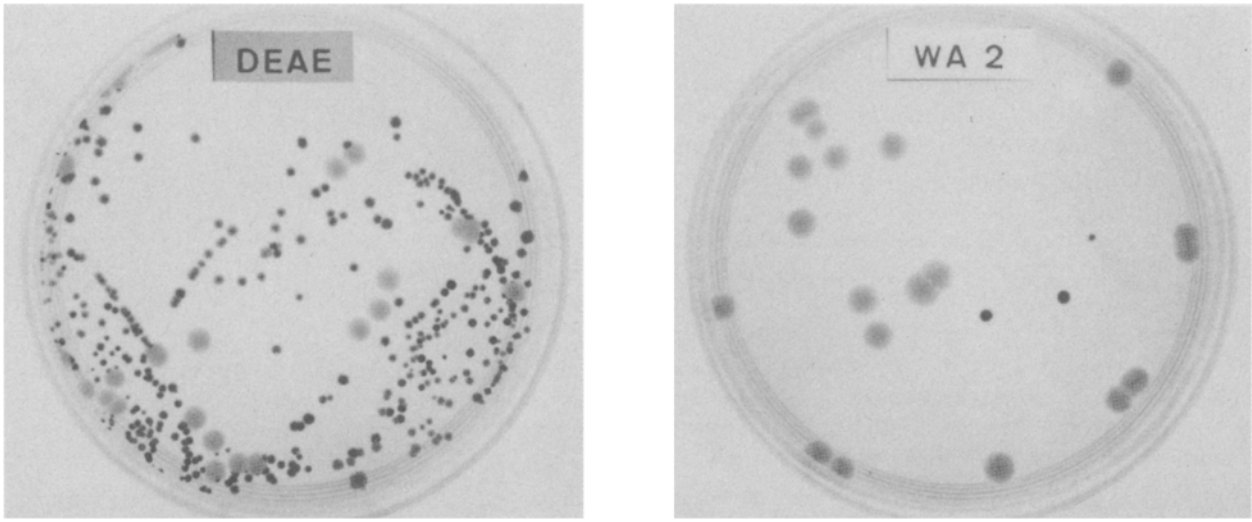


Fig. 4a and b. Plates with mixed cultures of *Rhizobium japonicum* st. 61-A-96 (big and light colonies) and *Chromobacterium violaceum* (small and dark colonies). (a) Desorbed from DEAE-cellulose paper; (b) desorbed from WA 2 paper

Table 4. Selection of *Rhizobium japonicum* st. 61-A-96 versus *Chromobacterium violaceum* from mixed cultures by ad- and desorption with ion-exchange papers. Incubation of papers in 5 ml suspension mixture with 3.1×10^9 cells (colony counting) of *Rhizobium japonicum* and 3.6×10^{11} cells (colony counting) of *Chromobacterium violaceum*. Ratio of rhizobia/chromobacteria: 0.0086

Ion exchange type	Blue colonies $\times 10^5$ *(Chromobacterium)* from 24 cm^2 IEP	White colonies $\times 10^5$ *(Rhizobium)* from 24 cm^2 IEP	Ratio rhizobia/chromobacteria
SB 2	4280	170	0.039
SA 2	5800	90	0.016
WB 2	3520	120	0.033
WA 2	680	480	*0.720*
CM-C	1160	60	0.054
DEAE-C	3280	80	0.026

plants for 15 min in 20 ml of a suspension of *Rhizobium japonicum* st. 61-A-96 (approximately 10^9 cells/ml) the roots were washed four times in successive 0.5 l of distilled water. Assuming, that about 1 ml of washing solution remained around the roots, the theoretical dilution factor without any adsorbance to the root surface would be about 6×10^{10}, 3 times more than *Rhizobium* cells were present in the total original suspension. The data in Table 5 show that a very significant number of rizobia are still attached to the roots, however, more are washed off by an incubation in distilled water but the number can be further increased by a factor of 2–3 by using 0.001 to 0.1 M NaCl or 0.005 M phosphate-buffer as desorbing solutions.

Table 5. Ad- and desorption of *Rhizobium japonicum* st. 61-A-96 from the root system of *Glycine max*

Desorbing solution	Desorbed living cells/plant[a]
Dist. water	1.76×10^6
0.001 M NaCl	4.5×10^6
0.01 M NaCl	4.7×10^6
0.1 M NaCl	5.4×10^6
0.005 M phosphate-buffer pH 5.5	4.6×10^6

[a] After a thoroughly washing of the root system with distilled water.

Discussion

The use of ion exchange papers has proved to be a fast and easy method to characterize different strains of *Rhizobium*. Between 10^6 and 10^7 cells could be bound to 1 cm^2 of the papers used. Since a single viable desorbed cell can be detected by plating and colony formation, the method has potentially a very high sensitivity. By using ^{32}P-labelled bacteria and counting the papers after desorption, we could also evaluate the number of bacteria remaining. We have no evidence if bacteria, that are desorbed by increasing ionic strength differ from those that are not detached. Their differential behaviour could simply be a question of their chance location on the paper at a more adhesive site. The adsorption (Table 1) shows a much higher specifity for the single bacterial strains than the desorption by using increasing ion concentrations (Figs. 1–3). By forming ratios of the cells, adsorbed to different types of ion exchange papers,

the discrimination between the strains is most evident. In some ratios, *Rhizobium lupini* differs by a factor of 10 from *Escherichia coli*, a *Rhizobium japonicum* strain by a factor of 5 from *Bacillus subtilis*. A specific selection and enrichment for *Rhizobium* has been demonstrated so far only against a small number of other bacterial strains.

With more information about the cationic-anionic charge pattern of *Rhizobium* strains, it might be possible to develop much more specific ion exchange surfaces *e.g.* by altering the spacing and the quality of the active groups. With such more specific surfaces it may be possible to select and enrich for rhizobia, *e.g.* from soil samples within minutes by a two or three fold ad/desorption step, compared to days or weeks with classical enrichment and selection methods.

The data in Table 5 indicate, that ad/desorption phenomena by ion-exchange could take also place in the root-*Rhizobium* interaction. We are still far from understanding the mechanism of action of several soil factors like H^+-concentration (van Schreven, 1972), Ca^{++}-availability (Lowther and Loneragan, 1970) and nitrate (Munns, 1968), in the complex nodulation process. Model studies with defined surfaces might also be helpful in understanding the possible interactions of the plant cell-bacterial cell surfaces and to separate these first steps from the later events. Lie (1969) reported the interesting fact, that pea roots developed in a neutral solution a mucous layer from pectinic substances, in which the rhizobia were embedded and which could serve as an ion-exchanger. A significant loss of the viability of *Rhizobium trifolii* by sodium chloride in peat was noted by Steinborn and Roughly (1974). The effective concentration of 0.2% (0.05 M) is about the same as that which resulted in or studies in a significant desorption from the ion-exchange papers. It is possible, that a similar desorption of rhizobia from the peat components by NaCl could be related to the reported loss of viability.

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References

Bergey's Manual of Determinative Bacteriology, R. E. Buchanan, N. E. Gibbons, eds. Baltimore: William and Wilkins 1974

Child, J. J.: Nitrogen fixation by a *Rhizobium* sp. in association with non-leguminous plant cell cultures. Nature (Lond.) **253**, 350–351 (1975)

Dart, P. J.: Development of root-nodule symbioses–The infection process. In: The biology of nitrogen fixation, A. Quispel, ed., pp. 381–429. Amsterdam-Oxford: North Holland 1974

Dorfner, K.: Ionenaustauscher, 2. Aufl. Berlin: de Gruyter 1964

Gibson, A. H., Dudman, W. F., Weaver, R. W., Horton, J. C., Anderson, I. C.: Variation within serogroup 123 of *Rhizobium japonicum*. Plant and Soil, Spec. **1971**, 33–37

Humphrey, B., Edgley, M., Vincent, J. M.: Absence of mannose in the extracellular polysaccharide of fast-growing *Rhizobia*. J. gen. Microbiol. **81**, 267–270 (1974)

Keele, B. B., Wheat, R. W., Elkan, G. H.: Carbohydrate, nucleic acid, and protein composition of extracellular material from *Rhizobium japonicum*. J. gen. appl. Microbiol. **20**, 187–196 (1974)

Lie, T. A.: The effect of low pH on different phases of nodule formation in pea plants. Plant and Soil **31**, 391–406 (1969)

Lowther, W. L., Loneragan, J. F.: Calcium in the nodulation of legumes. In: Proc. 11th Grassl. Congr., pp. 446–450. Queensland: Surfers Paradise 1970

Moffet, M. L., Colwell, R. R.: Adansonian analysis of the Rhizobiaceae. J. gen. Microbiol. **51**, 245–266 (1968)

Munns, D. N.: Nodulation of *Medicago sativa* in solution culture. Plant and Soil **29**, 257–262 (1968)

van Schreven, D. A.: On the resistance of effectiveness of *Rhizobium trifolii* to a low pH. Plant and Soil **37**, 49–55 (1972)

Snowcroft, W. R., Gibson, A. H.: Nitrogen fixation by *Rhizobium* associated with tobacco and cowpea cell cultures. Nature (Lond.) **253**, 351–352 (1975)

Steinborn, J., Roughly, R. J.: Sodium chloride as a cause of low numbers of *Rhizobium* in legume inoculants. J. appl. Bact. **37**, 93–99 (1974)

Trinick, M. J.: Symbiosis between *Rhizobium* and the non-legume *Trema aspera*. Nature (Lond.) **244**, 459–460 (1973)

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