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Immunotrapping of *Bacillus polymyxa* in Soil and in the Rhizosphere of Wheat*

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Abstract

A serological method for immunoisolation of *Bacillus polymyxa* from root-free soil and rhizosphere soil of wheat was developed. Microtiter plates were coated with rabbit polyclonal antibodies anti-*B. polymyxa* CF43 at 37°C for 3 hours. Root-free soil or rhizosphere soil was added and incubated at 4°C for 15 hours, and then microtiter plates were washed. The bound bacteria were desorbed with KCl 0.1 M pH 5.5 and plated on sucrose agar medium. Using classical method of enumeration, the population size of *B. polymyxa* in the rhizosphere of wheat is about 10^5 bacteria per g of soil, i.e. less than 0.1% of total microflora. When immunotrapping method was used as enrichment step, this percentage in root-free soil and in the rhizosphere soil fractions was respectively up to 20% and about 1%.

Introduction

The first description of a N_2-fixing anaerobe *B. polymyxa* was reported by Bredemann (1908). The confirmation of the N_2-fixing capacity of this bacteria, using ^{15}N occurred in 1958 (Hino and Wilson). Very little attention was paid to this N_2-fixing species, due to taxonomical problems (Rivière, 1963, Jurgensen and Davey, 1971), until the last 15 years. Today, the evidence of the association of *B. polymyxa* with the roots of wheat is well established (Lindberg and Granhall, 1984, Chanway et al., 1988, Heulin et al., 1990). In our laboratory, we are now interested in the comparison of biochemical and serological phenotypes of *B. polymyxa* populations isolated from (1) soil (root-free soil) and (2) from the rhizosphere of wheat, in order to

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investigate the effect of plant environment on the bacterial phenotypes. There is no problem for isolating well-adapted *B. polymyxa* in the rhizosphere of wheat, using the spermosphere model technique (Thomas-Bauzon et al., 1982). By contrast, it is necessary to improve another technique, due to lack of selective medium, for isolating *B. polymyxa* from root-free soil.

The immunotrapping technique, previously used in specific separation and isolation of human bone cells (Chess et al., 1974) was adapted to the isolation of bacteria from water, soil and plant samples (Hranitzky et al., 1980, Van Vuurde, 1987, Brunel et al., 1988). This technique consists in solid support coated with antibodies to trap bacteria recognized by these antibodies. In this work, immunotrapping technique was used for the isolation of *B. polymyxa* from root-free soil and rhizosphere soil.

Materials and Methods

Materials

Soil. Wheat seedlings (cv. Fidel) were grown both under laboratory (pots) and field conditions. Rhizosphere soil (root plus adhering soil) was sampled from the pots after a 12 days growth period and from the field after a 80 days cultivation period. Root-free soil was maintained in similar conditions to the corresponding rhizosphere soil (temperature, humidity). Soil extracts were obtained by suspending 5 g of macerated fresh soil samples in 45 ml of sterile phosphate-buffered saline (PBS) and stirring with a vortex mixer (Top-Mix 94323, Bioblock Scientific) at 2400 r.p.m. for 5 min. The above soil suspensions (10^{-1} dilution) were then diluted decimally in PBS.

Bacteria and media. The strain CF43 of *B. polymyxa* isolated from the rhizosphere of wheat (Heulin et al., 1990) was grown in Luria Bertani broth (Tryptone 10 g l^{-1}, yeast extract 5 g l^{-1}, NaCl 5 g l^{-1}). Sucrose agar medium was chosen for selection of *B. polymyxa*: sucrose 40 g l^{-1}, yeast extract 0.1 g l^{-1}, agar 15 g l^{-1}, supersalts solution 50 ml l^{-1} and phosphate buffer 15 ml l^{-1}. Supersalts solution and PBS were prepared as previously described (Heulin et al., 1987). The pH was adjusted to 6.8 and sterilized at 110°C for 20 min.

Immunoassay reagents and equipment. Coating buffer: Na-carbonate bicarbonate 0.1 M pH 9.5. Substrate buffer: diethanolamine 10% adjusted to pH 9.8 with HCl. Phosphate-buffered saline (PBS) pH 7.2: NaCl 7.2 g l^{-1}, Na_2HPO_4 2.8 g l^{-1}, KH_2PO_4 0.4 g l^{-1}. PBS-Tween: PBS plus 0.05% Tween 20. PBS-casein: PBS-Tween plus 0.5% casein. Microtiter plates: Costar 2596 (PolyLabo). Optical density of hydrolysed enzymatic substrate (4-nitrophenilphosphate ref. 107905, Boehringer Mannheim) was measured by spectrophotometer (Dynatech MR 700) at 405 nm.

Methods

Preparation of polyclonal antibodies (PcAbs). The rabbit antiserum used was prepared by A. Dorier (I.U.T. Biologie Appliquée, Lyon) against the strain CF43 of *B. polymyxa.* PcAbs were partially purified by precipitation with ammonium sulfate (3.9 M) and then dialysed against PBS.

PcAbs coating. Microtiter plates were washed overnight with ethanol. PcAbs anti-CF43 were coated at 37°C for 3 hours (0.1 ml per well). Plates were washed three times with 0.1 ml PBS-Tween and then incubated with 0.1 ml PBS-casein at room temperature for 30 min. Plates were washed twice with PBS-Tween and once with PBS (0.1 ml per well). The estimation of coated antibodies was made according to ELISA technique (Clark and Adams, 1977, Arsac and Cleyet Marel, 1986). Goat anti-rabbit IgG conjugated to alkaline phosphatase (GAR:ref. A7539 Sigma) diluted at 10^{-3} was added (0.1 ml per well) and incubated overnight at 30°C. Then paranitrophenilphosphate (1 mg ml^{-1} diethanolamine buffer) was applied (0.1 ml per well). Optical density was measured at 405 nm.

Immunotrapping assay. Microtiter plates were coated with partially purified rabbit PcAbs anti-CF43 as described above. Soil samples or bacterial suspension were added (0.1 ml per well). After incubation at 4°C for 15 hours, plates were washed twice with PBS (0.1 ml per well) and the bound bacteria were desorbed with either KCl 0.1 M pH 5.5 or PBS or Na-carbonate bicarbonate 0.1 M pH 8.5 or with distilled water pH 5.5. Mechanical treatment with pipette tips, used to dispense solutions, during few minutes was very important for effective desorption. Desorbed bacteria were plated on sucrose agar medium and incubated at 30°C. Typical production of exopolysaccharides by *B. polymyxa* made easy their selection.

Identification. B. polymyxa strains were identified by morphological and biochemical tests: cells rod shaped, rigid motility, ellipsoidal refractile spores located in subterminal position in swollen sporangia, Gram positif according to the Cerny's method (1976) and acidification of 49 carbohydrates using API 50 CH system (API-system, la Balme les Grottes-38390 Montalieu-Vercieu-France).

Results

Each step of immunotrapping technique was separately examined:

1. dilution of rabbit PcAbs anti-CF43 to coat microtiter plates,
2. chemical and physical treatments for desorption step,
3. incubation temperature of soil samples,
4. dilution of soil samples.

1. Dilution in carbonate bicarbonate (C–B) from 10^{-1} to 10^{-6} of rabbit PcAbs anti-CF43 were coated to microtiter plates (Costar 2596) and then goat anti-rabbit IgG conjugated to alkaline phosphatase was added (10^{-3} dilution). In these conditions the two optimal dilutions were 10^{-4} and 10^{-3}.

2. Microtiter plates were coated with rabbit PcAbs anti-CF43 (10^{-4} dilution) and dilutions of bacterial suspensions in PBS pH 7.2 were added. The best recovery yield was obtained with KCl 0.1 M pH 5.5 when 10^5 *B. polymyxa* CF43 per well were added (Fig. 1). IgG enzymatic digestions (papain, pepsin) or modification of hydrophobic bindings in presence of organic solvents did not increase the percentage of bacterial recovery (data not shown).

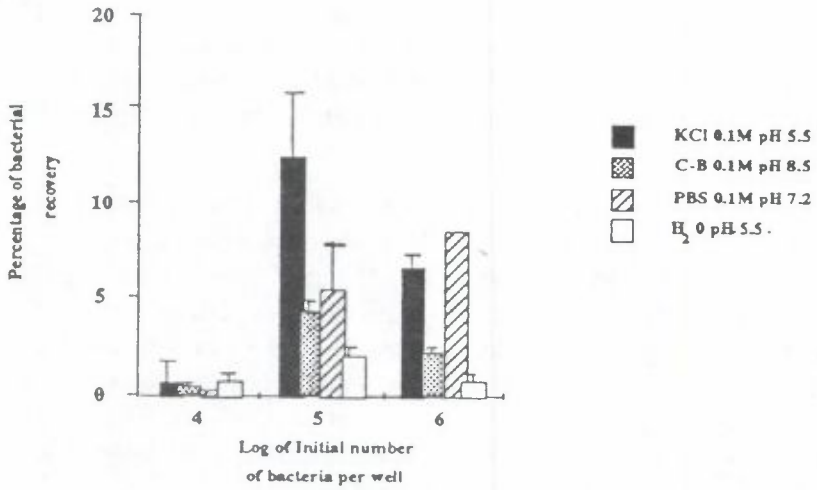

Figure 1. Determination of the optimal treatment for the desorption of attached *B. polymyxa* to rabbit PcAbs anti-CF43 (10^{-4} dilution) after plating on sucrose-agar medium.

Under optimal conditions (10^5 *B. polymyxa* CF43 per well, KCl 0.1 M pH 5.5), 12.5% of total bacteria were recovered after the first washing step, and less than 0.01% after two consequential washings. The control treatment consists in wells coated with Na-carbonate bicarbonate, without PcAbs. So, non-specific attachment of bacteria to the wells was about 0.05%, compared with 12.5% in presence of PcAbs in the wells.

3. Immunotrapping efficiency was affected by incubation temperature of microtiter plates containing root-free soil or rhizosphere soil samples. In both cases, total number of immunotrapped bacteria was higher when incubation temperature was 30°C (Table 1). This temperature, applied during 15 hours, results in a selection of the fast-growing bacteria (*Enterobacteriaceae*, *Pseudomonas*). This ten fold increase of

bacterial population size during incubation at 30°C was inconsistent with isolation of *B. polymyxa*. At 4°C, the percentage of recovered *B. polymyxa* after plating on sucrose agar medium was 13% of total immunotrapped bacteria in wells, only for the root-free soil fraction. When using rhizosphere soil, this percentage of *B. polymyxa* was about 1% of total immunotrapped bacteria.

Table 1. Effect of incubation temperature of root-free soil and rhizosphere soil of a 12-day-old wheat (cv. Field) on immunotrappling efficiency of *B. polymyxa*

Samples	Incubation temperatures	Number of recovered bacteria ($\times 10^6$ gdw^{-1})	Total *Bacillus* (% recov. bact.)	Recovered *B. polymyxa* ($\times 10^5$ cfu gdw^{-1})	(%)
Root-free soil	4°C	0.2±0.08	60±11	0.2±0.1	13±11
	30°C	3.7±0.2	1.0±0.9	0	0
Rhizosphere soil	4°C	32±24	3±1	4±1.3	1.0±0.3
	30°C	380±110	3±4	0	0

gdw^{-1}:per g dry weight of soil at 60°C for 48 h.

4. The determination of the best soil sample dilutions was made with a 10^{-3} of rabbit PcAbs anti-CF43 at 4°C and using KCl 0.1 M pH 5.5 for the desorption step. Once more, the best efficiency of the method was observed with root-free soil sample, up to 22% of total immunotrapped bacteria, when 10^{-3} dilution of soil is used (Table 2). With the rhizosphere soil sample, this percentage was lower than 1%, whatever the dilutions used.

Table 2. Effect of serial dilutions of root-free soil and rhizosphere soil of wheat (cf. Fidel) at grain maturation stage on immunotrapping efficiency of *B. polymyxa*

Samples	Dilutions	Number of recovered bacteria ($\times 10^5$ gdw^{-1})	Total *Bacillus* (% recov. bact.)	Recovered *B. polymyxa* (10^4 cfu gdw^{-1})	(%)
Root-free soil	10^{-1}	7.8±0.1	64±24	0.1±0.02	1.2±0.8
	10^{-2}	4.9±0.4	94±2	0.4±0.2	0.4±0.7
	10^{-3}	3.9±0.2	83±8	4.2±1.3	22.0±15.0
Rhizosphere soil	10^{-1}	ND	ND	ND	ND
	10^{-2}	5.7±1.5	7±4	0	0
	10^{-3}	22.0±15.0	6±5	0	0

Discussion and Conclusion

In root free soil, the population size of *B. polymyxa* was about 0.2 to 0.5×10^5 bacteria per g dry weight of soil (Table 1 and 2), corresponding to 0.1% of total microflora. Using immunotrapping technique as enrichment step, this percentage of *B. polymyxa* was between 10 to 20% (Table 1 and 2). The stimulation factor of the

method was about 100 fold. The estimation of the population size of *B. polymyxa* in the rhizosphere of wheat, 4×10^5 bacteria per g dry weight of soil (Table 1), is in agreement with literature data (Heulin et al., 1990) between 1 to 5×10^5 bacteria per g dry weight of soil. According to these authors, the percentage of *B. polymyxa* in this environment is also about 0.1% of total microflora. But here, the immunotrapping method was not so efficient compared with the root-free soil: only 1% of the immunotrapped bacteria were *B. polymyxa* (10 fold stimulation).

The immunotrapping method described here is suitable for isolation of *B. polymyxa* from root-free soil fraction, outside the rhizosphere. It was one of the main objective of this work. With rhizosphere soil samples, the efficiency of the method was not as satisfactory as with root-free samples, probably due to higher dilution of *B. polymyxa* among fast-growing bacteria in the rhizosphere of wheat. Phenotypical and serological diversity of representative populations of *B. polymyxa*, isolated in and outside the rhizosphere of wheat will be investigated.

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