ANNALES
UNIVERSITATIS MARIAE CURIE-SKŁODOWSKA
LUBLIN — POLONIA

VOL. XLVIII, 1 SECTIO C 1993

Instytut Mikrobiologii UMCS
Zakład Mikrobiologii Ogólnej

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Effect of Acidic Conditions on Growth and Nodulation of *Rhizobium leguminosarum* bv. *trifolii*

Wpływ warunków kwaśnych na wzrost i brodawkowanie *Rhizobium leguminosarum* bv. *trifolii*

Bacteria of the genus *Rhizobium* induce nodules and fix nitrogen in the roots of leguminous plants. The formation of nitrogen-fixing nodules by bacteria is a complex process that begins with proliferation of *Rhizobium* and induction of common nod genes.

Environmental factors such as soil acidity influence the effectiveness of symbiosis. *Rhizobium leguminosarum* bv. *trifolii*, which infects *Trifolium* is acid sensitive in either broth cultures or soil. The acidic conditions have also negative effect on the induction of the nod genes by root exudates. Much of the usable land of the world is acidic, and acidity is a major factor which limits the legume crop. The toxic effect of acidic conditions is due partly to the higher solubility of some cations (Al, Mn, Fe) in low pH. So, it is difficult to separate the toxicity effects of Al and low pH, because they often occur together. Therefore, studies on the effect of low pH and metal ions on survival and nodulation by rhizobia are important from practical point of view.

MATERIALS AND METHODS

Bacterial strains used in this study are listed in Table 1. Mannitol-yeast extract medium (79 CA) was used as complete medium , M1 medium was used as minimal medium for *Rhizobium*.

The nodulation tube-test was performed as described by Vincent (11). Surface sterilized seeds of *Trifolium pratense* were germinated on nitrogen-free R medium. The

5-day-old seedlings were transferred to sterile slants of the same medium with different *pH* and inoculated with bacteria. Ten replicates for each bacterial strain were used.

Plant exudates preparation: lucerne seeds were surface sterilized and incubated on R medium for 4 days. Then, the seed exudates were collected and sterilized by boiling for 5 min.

Assay of *pH* effect on bacteria: bacteria were growing on 79 CA agar plates at *pH* adjusted to: 3.0, 4.0, 5.0, 6.0, 7.0, 8.0 at 28°C for 48 h. The plates were inoculated with approximately 1000 cfu per 1 ml from diluted 1 day old liquid cultures.

Test of induction of nodC-lacZ gene: bacterial cultures were grown to OD of 0.2 at 550 nm and 50 μl of plant exudates were added to 2.5 ml samples for induction. The cultures were incubated with shaking for 4 h. β-galactosidase activity was assayed using the O-nitrophenyl-β-D-galactoside (ONPG) as described by Miller (4). Determination of poly-β- hydroxybutyrate (PHB): the procedures for isolation and quantitative estimation of PHB as described by Karr et al.(3) were applied to bacteria R.t. AR20 and parental strain R.t.24.1 Degradation activity of PHB was determined as clear zone appearance around positive colonies in M1 medium supplemented with PHB.

Determination of iron-regulated proteins in outer membrane: bacteria were growing in M1 and M1 with 20 μM of Fe to stationary phase (Fig. 1). Outer membranes were obtained according to Reigh and O'Connell (7) with further purification on linear 25–60% saccharose gradient (2). The samples were loaded on SDS — 12.5% polyacrylamide gels, which were run at 30 mA in buffer at *pH* 8.4. The gels were stained with Coomasie blue.

RESULTS AND DISCUSSION

We have studied the influence of the different *pH* on the growth and nodulation of *Rhizobium leguminosarum* bv. *trifolii* 24 (R.t.24) and its symbiotic mutants: AR6, AR16, AR20 and R.t.93 (Table 1). Strains R.t.93, AR16 and AR20 do not produce acidic exopolysaccharide (EPS). AR20 has also altered lipopolysacharide. AR6 is able to produce the phenolate type of siderophore (9, 10). All the substances (EPS, LPS and siderophores) bind metal ions, thus can change response of bacteria to low *pH*. *R. leguminosarum* bv. *trifolii* AR20 has another metabolic defect, it accumulates large amount of PHB, being unable to degrade this polymer.The results of *pH* — growth dependence indicate that for tested strains optimal growth was at slightly acidic *pH*. The only strain able to grow at very low *pH* (4.0) was AR6 (Table 2).

For establishing relationship between optimal *pH* and nodulation abilities the above strains were used for infection of clover plants grown in slightly acidic *pH* and neutral values (Table 3). A general feature of the strains derived from R.t.24 was a few days delayed nodulation as compared to the parental strain . At the end of experiment the lowest number of nodules per plant showed the strain AR6 and R.t.24, these values were *pH*-independent.

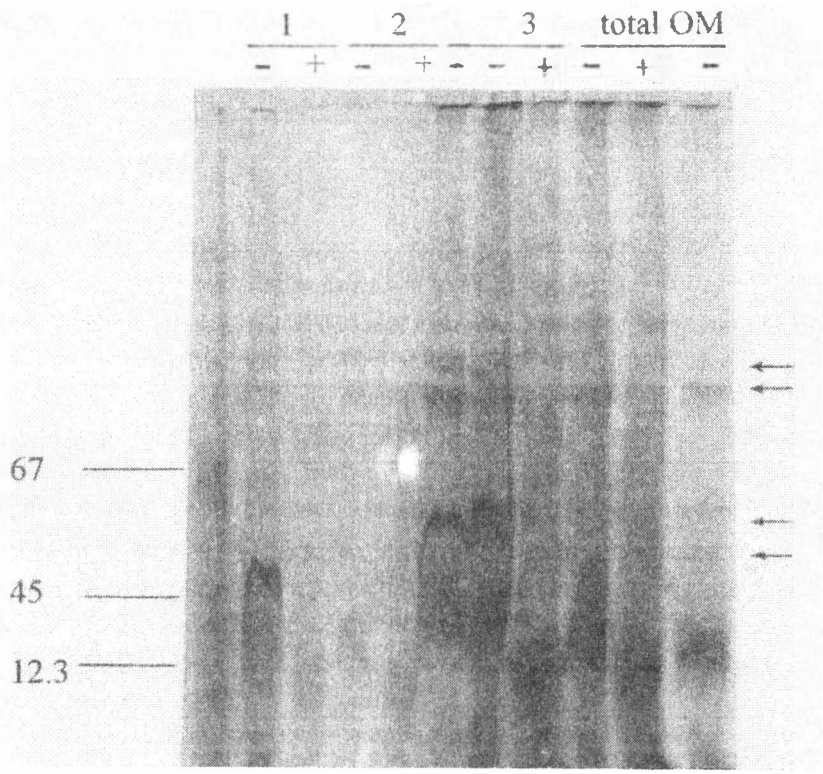

Fig. 1. Outer membrane profiles of *Rhizobium leguminosarum* bv. *trifolii* AR6 grown in low-iron minimal medium (−) and the medium supplemented with 20 μM of iron (+). Lanes: 1, 2 and 3 are the fractions of the same density from saccharose gradient of outer membrane isolated from the cultures without (−) and (+) iron added. Total OM-proteins not separated. Molecular sizes in kilodaltons (kDa) are shown on the left. Arrowheads indicate the bands of proteins induced by iron starvation

Table 1. The characteristics of the strains of *Rhizobium leguminosarum*

Strain	Characteristics
R.t.24	Nod+ Fix+ Exo+
R.t.93	Nod+ Fix− Exo−
AR 16	Nod+ Fix− Exo−
AR 20	Nod+ Fix− Exo−
AR 6*	Nod+ Fix− Exo− Sid+

Explanation: Nod — ability to nodule formation; Fix — ability to nitrogen fixation; Exo — capability for the acidic exopolysaccharide production; Sid^+ — ability to siderophore production.

* Forms ineffective nodules when a large inoculum is used.

Table 2. Growth of wild-type and exo- and muc- strains on the yeast extract-mannitol agar at different starting *pH* values

Strain	optimal *pH*	minimal *pH*	maximal *pH*
R.t.24.1	6	4	8
R.t.93	5–6	4	8
R.t. AR16	5–6	4	7.5
R.t. AR20	5–6	4	7.5
R.t. AR6	5–6	3	8

Table 3. Nodulation of white clover by *Rhizobium leguminosarum* bv. *trifolii* strains at different starting *pH* values

Strain	*pH*	Nodules per plant after n-days from infection			
		6 days	12 days	22 days	44 days
R.t.24	5.3	0.3	2.3	4.8	5.3
	6.3	0.5	2.7	3.6	4.8
	7.2	0.6	2.5	3.3	4.6
R.t.93	5.3	0	1.5	5.0	10.0
	6.3	0	1.4	6.0	13.0
	7.2	0	0.4	1.7	4.6
AR 16	5.3	0	1.4	6.0	13.0
	6.3	0	0.6	6.3	10.0
	7.2	0	0.5	7.5	11.0
AR 20	5.3	0	0.4	6.0	9.0
	6.3	0	0.6	4.7	10.6
	7.2	0	0.5	5.4	12.4
AR 6	5.3	0	2.0	3.0	4.6
	6.3	0.3	2.3	4.0	5.3
	7.2	0	1.2	4.2	5.2

As was mentioned (Table 1) strain AR6 forms ineffective nodules only when very large inoculum was used. One possible explanation is that this strain shows low level of nodC gene induction in presence of root exudates (Table 5). Therefore, the production of signal substance by AR6 is strongly reduced. Conversely, the strains AR16 and AR20 formed 2–3 times more nodules than parental strain. The only strain, which exhibited *pH*-dependent nodule formation was mutant R.t. 93. This strain forms earlier nodules at acidic *pH*. It forms two times higher number of nodules at lower *pH* as determined at the end of experiment as compared to those at neutral *pH*. However, the enhanced ability to nodule formation was not accompanied with restoration of nitrogen fixation. The induction of expression of nodC gene in strain R.t.93 is the same at *pH* 7 and 6, but it is reduced at *pH* 5 (Table 4).

Table 4. Effect of *pH* on growth of *Rhizobium trifolii* 93 and nodC-lacZ induction by root exudate (re)

pH	Growth (OD_{550})		β-galactosidase activity (u)[1]		
	-re	+re[2]	-re	+re[2]	Induction index[3]
7	0.48	0.38	25	359	14.3
6	0.52	0.35	29	390	13.4
5	0.6	0.31	27	87	3.2

Explanation: 1 — measured as described by Miller (4), 2 — 50 μl of alfalfa root exudate were added to 2.5 ml of culture, 3 — ratio of β-galactosidase activity in presence of root exudate to this activity in the absence of root exudate.

Reed and Walker (6) found that a slightly acidic *pH* restored the symbiotic properties of exoD mutants of *R. meliloti*. Richardson et al. found (8) expression of nodulation genes in *Rhizobium leguminosarum* biovar *trifolii* is affected by low *pH*.

In our previous papers (9, 10) it was demonstrated that AR6 produced the phenolate-type of siderophore. In this paper the outer membrane (OM) profiles of this strain grown under high- and low- iron conditions were examined. As it was shown in Fig.1 there is induction of some proteins under low-iron conditions. Two apparently visible protein bands of approximately 80 kDal were observed in total OM proteins before their saccharose fractionation. Two other less intense minor bands of approximately 50 kDa were visible in total OM preparation . It has been known that an iron inducible protein pattern is strain specific (5, 7). The availability of iron is a factor that could change the expression of nod genes.We found that iron at concentration 1–10 μM increased nodC induction in strain R.t.93 and R.t.24. There was no such effect on strain AR6 which produced siderophore (Table 5).

Table 5. Effect of iron on nodC-lacZ induction

Concentration of iron	R.t.24		R.t.93		AR6	
	U	I	U	I	U	I
Control, -re	11.2	—	10.6	—	38	—
0	243	21.6	230	21.6	106	2.8
1	518	46.2	545	51.4	135	3.5
5	582	51.9	574	54.1	140	3.6
10	672	60.0	697	65.7	136	3.5

See legend for Table 4.

In conclusion, the response of bacteria to low *pH* and some cations depends on their ability to produce siderophore and EPS. These substances could change both bacterial growth and nodulation in low *pH*.

REFERENCES

1. Chen H., Richardson A. E., Gartner E., Djordjevic M. A., Roughley B. J., Rolfe B. G.: Construction of an acid-tolerant *Rhizobium leguminosarum* biovar *trifolii* strain with enhanced capacity for nitrogen fixation. Appl. Environ. Microbiol. **57**, 2005–2011 (1991).
2. Hubac C., Guerrier D., Ferran J., Tremolieres A., Kondorosi A.: Lipid and protein composition of outer and inner membranes in wild type strains and nod mutants of *Rhizobium meliloti*. J. Gen. Microbiol. **138**, 1973–1983 (1992).
3. Karr D. B., Waters J. K., Emerich D. W.: Analysis of poly-β-hydroxybutyrate in *Rhizobium japonicum* bacteroids by ion-exclusion high-pressure liquid chromatography and UV detection. Appl. Environ. Microbiol. **46**, 1339–1344 (1983).
4. Miller J. H.: Experiments in Molecular Genetic. Cold Spring Harbor Lab., Cold Spring Harbor, N.Y. 1972.
5. Neilands J. B.: Microbial iron compounds. Ann. Rev. Biochem. **50**, 715–731 (1981).
6. Reed J. W., Walker G. C.: Acidic conditions permit effective nodulation of alfalfa by invasion-deficient *Rhizobium meliloti* exoD mutants. Genes Dev. **5**, 2274–2287 (1991).
7. Reigh G., O'Connell M.: Siderophore-mediated iron transport correlates with the presence of specific iron-regulated proteins in the outer membrane of *Rhizobium meliloti*. J. Bacteriol. **175**, 94–102 (1993).
8. Richardson A. E., Simpson R. J., Djordjevic M. A., Rolfe B. G.: Expression of nodulation genes in *Rhizobium leguminosarum* biovar *trifolii* is affected by low *pH* and Ca and Al ions. J. Bacteriol. **54**, 2541–2548 (1988).
9. Skorupska A., Choma A., Deryło M., Lorkiewicz Z.: Siderophore containing 2,3-dihydroxybenzoic acid and threonine formed by *Rhizobium trifolii*. Acta Biochim. Polon. **35**, 119–130 (1988).
10. Skorupska A., Deryło M., Lorkiewicz Z.: Siderophore production and utilization by *Rhizobium trifolii*. Biol. Metals. **2**, 45–49 (1989).
11. Vincent J. M.: A Manual for the Practical Study of Root Nodule Bacteria. Handbook no. 15. Blackwell Sci. Publ. Oxford 1970.

STRESZCZENIE

Badano wpływ niskiego *pH* na wzrost, brodawkowanie i ekspresję genów nod odpowiedzialnych za brodawkowanie u *Rhizobium leguminosarum* biovar *trifolii* 24 i jego symbiotycznych mutantów. Wykazano, że optymalne *pH* dla wzrostu badanych szczepów wynosiło około 6. Wszystkie szczepy brodawkowały z jednakową wydajnością w zakresie *pH* 5–7, z wyjątkiem szczepu R.t. 93, który tworzył większą liczbę brodawek w *pH* 5. Jednak nawet w tym ostatnim szczepie wyrażenie genu nodC było znacznie zredukowane w *pH* 5. Szczep AR6, produkujący siderofor, rósł w najszerszym zakresie *pH* (4–8). W *R. leguminosarum* bv. *trifolii* AR6 wykryto w membranie zewnętrznej białka indukowane głodzeniem żelazowym.