ABSENCE OF A CORRELATION BETWEEN NITRATE REDUCTASE AND SYMBIOTIC NITROGEN FIXATION EFFICIENCY IN RHIZOBIUM MELILOTI

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Specific activities of the assimilatory and "regulatory" types of nitrate reductase were studied in 41 strains of *Rhizobium meliloti* having different symbiotic nitrogen fixation activities. Both nitrate reductase enzymes were present in very effective and ineffective strains and no significant correlation was found between the specific activities of the two enzymes and the dry matter yields of alfalfa obtained with the 41 strains. Measurements of the specific activities of the two nitrate reductase enzymes in the vegetative bacteria cannot be used as a rapid physiological test for the selection of very effective strains of *R. meliloti*.

Les activités spécifiques des nitrates réductases assimilatoires et "régulatoires" ont été étudiées chez 41 souches de *Rhizobium meliloti* ayant des activités différentes de fixation symbiotique d'azote. Les deux nitrates réductases ont été retrouvées chez les souches très efficaces ainsi que chez les souches inefficaces et aucune corrélation significative n'a été trouvée entre les activités spécifiques et le rendement de la matière sèche de la luzerne obtenu avec les 41 souches à l'essai. La détermination des activités spécifiques de ces deux nitrates réductases chez les bactéries végétatives ne peut donc pas être utilisée comme un test physiologique rapide pour la sélection de souches très efficaces de *R. meliloti*.

The presence of a significant correlation between nitrate reductase and nitrogenase enzyme activities was suggested in *Rhizobium japonicum* (Cheniae and Evans 1960) and *Rhizobium meliloti* (Kondorosi et al. 1973; Sik et al. 1976). Evans and Russel (1971) also suggested that the two enzymes share a common structural protein component. A mutation in the suggested common component should have a pleiotropic effect on both nitrate reductase and nitrogenase. By using three ineffective mutants of *Rhizobium meliloti*, Sik and Barabás (1977) showed that the assimilatory type of nitrate reductase (produced when nitrate is the only nitrogen source in the culture medium) could be induced in the effective wild-type bacteria as well as in its ineffective mutants, and they concluded that the connection between the two reducing enzymes is not of a structural nature. However, a special type of nitrate reductase labelled "regulatory" was detected only in the effective wild-type bacteria, cultured under aeration on amino acids both as nitrogen and carbon sources. A possible regulatory connection between the "regulatory" type of nitrate reductase and nitrogenase was proposed (Barabás and Sik 1979; Sik and Barabás 1977). If this hypothesis, based on observations made with a few mutants, could be applied to the

symbiotic nitrogen fixation systems of a relatively larger population of *R. meliloti*, then a positive significant correlation is to be expected between the specific activity of the "regulatory" nitrate reductase and the symbiotic nitrogen fixation efficiency, and a rapid physiological test for the selection of very effective strains will be established. Because the selection of very effective strains of *Rhizobium* is a time-consuming process (Bordeleau et al. 1977), there is an increasing need for rapid and practical methods of strain selection (Bordeleau and Antoun 1978).

In the present work, the specific activities of the assimilatory and "regulatory" types of nitrate reductase were studied in 41 strains of *R. meliloti* having different symbiotic nitrogen fixation activities.

MATERIALS AND METHODS

Bacteria

The 41 strains of *Rhizobium meliloti* used in this study and their symbiotic nitrogen fixation efficiency were described in a previous report (Bordeleau et al. 1977).

Assimilatory Nitrate Reductase

This enzyme is formed in bacteria growing with nitrate as the only nitrogen source (Sik and Barabás 1977). Bacteria were cultured, at 26.6°C during 7 days on a rotary shaker, in a liquid medium containing per liter of distilled water: mannitol, 10.0 g; KNO_3, 1.0 g; NaCl, 0.1 g; K_2HPO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; yeast extract (Difco), 0.1 g; *p*H, 7.0. Bacterial cells from 10 mL of the suspension were washed in saline and digested at 100°C in 1 mL of 1 N NaOH for 10–20 min. After neutralization with 1 N HCl, the protein content was measured by the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, Calif.) based on the method of Bradford (1976). Residual nitrate in the liquid supernatant was measured by the method of Cataldo et al. (1975) and the nitrite formed was determined as described by Bremner (1965). The specific enzyme activity was expressed as the amount of nitrate reduced or nitrite detected per microgram of protein.

"Regulatory" Nitrate Reductase

This type of enzyme is detectable in bacteria grown on amino acids both as nitrogen and carbon sources, without added nitrate, and it was suggested that it is induced by endogenous formation of nitrate (Sik and Barabás 1977). Bacteria were cultured for 48 h at 26.6°C on a rotary shaker, in YTB medium (Orosz and Sik 1970) which contained per liter of distilled water: tryptone (Difco), 10.0 g; yeast extract (Difco), 1.0 g; NaCl, 5.0 g; $CaCl_2 \cdot 2H_2O$, 0.15 g; $MgSO_4 \cdot 7H_2O$, 0.25 g; *p*H, 7.0. Bacterial cells were washed in saline and resuspended in fresh YTB medium and incubated for 2.5 h at 26.6°C on a rotary shaker. Cells from 5 mL of this suspension were collected and resuspended in a buffered saline solution (NaCl, 7.0 g; KH_2PO_4, 0.34 g; K_2HPO_4, 1.21 g; *p*H, 7.2). To 1-mL samples, 10 mmoles nitrate was added and after incubation for 10 or 20 min at 30°C without aeration, nitrite was determined as described by Nicholas and Nason (1957). The specific enzyme activity was expressed as the amount of nitrite produced per microgram of protein per minute.

RESULTS AND DISCUSSION

All the tested strains of *R. meliloti* reduced nitrate except the effective strain 3DOa2oa. This strain failed to utilize nitrate and showed slight growth on yeast extract added as vitamin source in the culture medium (Table 1). The highest nitrate reduction was performed by the effective strain 23A. The nitrite detected in the culture medium measures the nitrate reductase activity but also reflects the nitrite reductase activity. In fact, one might expect to detect more nitrite if a stain has a high nitrate reductase activity and/or a low nitrite reductase activity. The very effective strain A_3 reduced nitrate but no nitrite was detected, indicating a high activity of nitrite reductase. The highest amount of nitrite was detected with the effective strain A_5, indicating a high activity of nitrate reductase and also a lower activity of nitrite reductase if compared to strain 23A, for example (Table 1). The two effective strains S_8 and S_9 had assimilatory nitrate reductase activity but did not show any "regulatory" nitrate reductase activity. The highest "regulatory" nitrate reductase activity was recorded with the effective strain S_{19} (Table 1). All very effective and

Table 1. Assimilatory and regulatory nitrate reductase (NR) activities in *Rhizobium meliloti*

Strains	Assimilatory NR: nanog $N\text{-}NO_3$ reduced per μg protein	Assimilatory NR: nanog $N\text{-}NO_2$ detected per μg protein	Regulatory NR: picog $N\text{-}NO_2$ (μg protein)$^{-1}$min^{-1}	Efficiency†
S_1	572.2	44.95	31.50	E
S_2	617.0	1.91	14.00	E
S_3	274.2	0.68	10.78	E
S_4	271.4	1.32	18.06	E
S_5	329.2	0.36	14.42	E
S_6	41.6	1.83	18.90	E
S_7	217.2	0.38	9.38	E
S_8	230.3	0.39	0	E
S_9	94.3	6.98	0	E
S_{11}	256.3	6.35	11.62	E
S_{12}	300.0	0.47	10.78	E
S_{14}	248.5	2.66	57.12	VE
S_{15}	323.6	9.28	22.68	E
S_{16}	372.8	3.49	111.72	E
S_{19}	368.3	4.27	145.88	E
S_{20}	302.1	0.86	61.74	E
S_{21}	429.1	12.37	5.88	E
S_{22}	390.0	1.66	73.64	E
V_1	272.5	4.95	43.82	E
V_2	368.5	79.74	58.38	E
V_3	219.8	4.80	51.10	VE
V_4	496.6	10.66	8.82	E
D_1	477.7	11.98	73.22	I
D_2	1168.1	154.54	52.36	E
D_3	381.7	10.37	3.64	E
A_2	355.9	2.88	50.82	VE
A_3	408.7	0	1.40	VE
A_4	360.8	16.98	1.54	VE
A_5	1292.1	168.44	20.16	E
I_1	32.0	4.23	27.44	I
I_2	518.4	5.26	33.74	I
I_3	382.1	4.44	21.56	E
I_4	291.1	2.08	27.16	I
R_1	507.0	4.06	21.70	E
E_2	319.1	1.96	5.74	E
23A	1325.2	84.51	32.48	E
3DOa2oa	0	0	8.40	E
3DOa8	479.8	15.96	4.06	VE
54032	510.7	5.80	29.12	E
54033	251.4	4.55	10.64	E
Alfalfa D	580.3	12.26	75.88	I

†Based on yields obtained in the second cutting (Bordeleau et al. 1977). VE, very effective; E, effective; I, ineffective.

ineffective strains had assimilatory and "regulatory" nitrate reductase activities. This indicates that no relationship exists between the nitrate reductase activities and the symbiotic nitrogen fixation activity. In fact, statistical analysis did not show any significant coefficient of correlation between the nitrate reductase activities and the dry matter yields of alfalfa (Table 2) which reflects the symbiotic nitrogen fixation activity of the tested strains (Bordeleau et al. 1977). The presence of assimilatory nitrate

Table 2. Coefficients of correlation (r) between nitrate reductase (NR) activity of *Rhizobium meliloti* and its nitrogenase activity with alfalfa

	Nitrogenase activity†		
	1	2	1+2
Assimilatory NR			
NO_3 reduced	−0.14NS‡	0.03NS	−0.01NS
NO_2 detected	−0.04NS	−0.05NS	−0.03NS
"Regulatory" NR	−0.08NS	−0.14NS	−0.13NS

†Nitrogenase activity expressed as alfalfa dry matter yield obtained at first cutting (1) and second cutting (2).
‡NS, not significant.

reductase in the very effective and ineffective strains (Table 1) confirms the previous observations made on ineffective mutants by Sik and Barabás (1977) that there is no structural connection between nitrate reductase and nitrogenase.

Sik and Barabás (1977) detected the "regulatory" nitrate reductase only in the effective wild type *R. meliloti*, and because this enzyme was absent in the ineffective mutants they suggested the presence of a common regulation factor governing the activities of this type of nitrate reductase and nitrogenase. In this study, the "regulatory" nitrate reductase was detected in both very effective and ineffective strains. This indicates that there is no common direct regulation factor governing the activities of the "regulatory" nitrate reductase and the symbiotic nitrogen fixation activities in the 41 tested strains. From these results, we can also conclude that the specific activities of the assimilatory and "regulatory" types of nitrate reductase do not offer useful physiological tools for rapid selection of very effective strains of *R. meliloti*.

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