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NITROGENASE ACTIVITY IN PURE CULTURES OF SPECTINOMYCIN-RESISTANT FAST AND SLOW-GROWING *RHIZOBIUM*

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SUMMARY: Several strains of *Rhizobium* resistant to spectinomycin also had nitrogenase activity (C_2H_2 reduction and H_2 production) in static culture under 95% Ar/1%O_2/4%C_2H_2. This relationship between nitrogenase activity and spectinomycin resistance was observed in both fast-growing (*R. trifolii* and *R. leguminosarum*) and slow-growing (*R. japonicum*) rhizobia. The effect of different media and various carbon sources on nitrogenase activity was investigated in more detail in *R. trifolii* strain T1Sp. This communication demonstrates that fast-growing rhizobia can have nitrogenase activity in the absence of any plant component.

INTRODUCTION

Rhizobium, a genus of bacteria which form symbiotic nitrogen-fixing nodules on roots of legumes is commonly sub-divided into two groups based mainly on rate of growth. It is now well established that certain strains of the slow-growing rhizobia, (*R. japonicum* and the cowpea group) can fix nitrogen when cultured in the absence of any plant material (1, 2, 3, 4, 5). However, a repeatable and consistent nitrogenase activity has not been found for the fast-growing rhizobia, although two reports have described an acetylene reducing activity in one strain of *R. leguminosarum* (5) and another strain of *R. trifolii* (6).

Recently, nitrogenase activity has been elicited in both fast- and slow-growing rhizobia by a conditioned medium obtained from suspension cultures of soybean (*Glycine max* L. Merrit) cells after several days incubation with rhizobia (7, 8).

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This report describes a correlation between resistance to the antibiotic spectinomycin and the presence of nitrogenase activity in fast-growing rhizobial strains.

MATERIALS AND METHODS

Bacterial strains: *R. trifolii* strain Tl and a spectinomycin-resistant (Sp^R) derivative, TlSp, were used. Two further Sp^R derivatives, RT52 and RT53, were isolated from strain Tl after EMS mutagenesis, and were resistant to 100 µg/ml spectinomycin. *R. leguminosarum* strain L4Sp^R was also used. Sp^R spontaneous mutants were also isolated in the slow-growing *R. japonicum* strain 3I-1b-138, and the one studied in detail was designated RJ1.

Media: Vincent's medium (VYM) (9) contained per litre, mannitol 10 g, yeast extract 0.4g, $MgSO_4.7H_2O$, 0.2 g, NaCl 0.1 g, K_2HPO_4 0.25 g. Valera and Alexander's medium (V & A) (10) contained per litre, sodium gluconate 10 g, inositol 0.01 g, casein hydrolysate 0.5 g, $(NH_4)_2SO_4$ 0.065 g, K_2HPO_4 0.8 g, KH_2PO_4 0.2 g, $MgSO_4.7H_2O$ 0.4 g, $CaCl_2.2H_2O$ 0.1 g, $FeCl_3.6H_2O$ 0.01 g, $Na_2MoO_4.2H_2O$ 0.2 mg, $ZnSO_4.7H_2O$ 0.2 mg, H_3BO_3 0.2 mg, $MnSO_4.H_2O$ 0.2 mg, *p*-aminobenzoic acid 0.1 mg, pyridoxine HCl 0.1 mg, thiamine HCl 1 mg, Ca pantothenate 1 mg, riboflavin 1 mg, pyridoxal HCl 1 mg, $CuSO_4$ 15 µg, $CoCl_2.6H_2O$ 1 µg. Bergersen's modified medium (BMM) (11) contained per litre, mannitol 10 g, glutamate 0.5 g, yeast extract 0.5 g, $Na_2HPO_4.12H_2O$ 0.36 g, $MgSO_4.7H_2O$ 0.08 g, $FeCl_3.6H_2O$ 3 mg, $CaCl_2.2H_2O$ 40 mg, thiamine HCl 100 µg, biotin 50 µg, Gamborg's trace elements (12) 1 ml. Nitrogen-free B5 medium (NNB5) (12) contained per litre, sucrose 20g, $NaH_2PO_4.2H_2O$ 1.5 g, KI 7.5 mg, KCl 5 g, Na_2SO_4 1.5 g, $MgSO_4.7H_2O$ 2.5 g, $CaCl_2.2H_2O$ 1.5 g, inositol 10 mg, thiamine HCl 1 mg, nicotinic acid 0.1 mg, pyridoxine HCl 0.1 mg, Gamborg's trace elements (12) 1 ml, Fe-EDTA 0.5 ml. Spectinomycin (Upjohn) was filter-sterilized and used in media at a final concentration of 100 µg/ml.

Nitrogenase assays: Cultures were inoculated from BMM plates containing 100 µg/ml spectinomycin into the particular liquid medium and shaken at 250 rpm at 30°C for 2 to 4 days prior to use. 1 ml of the cell suspension was then added, with 1 ml of dilution medium (NNB5 or V & A) and an added carbon source (20 mM final concentration) to vials. The vials were capped with serum stoppers, evacuated and flushed four times with a 95% Ar/1%O_2/4%C_2H_2 gas mixture, and incubated at 30°C (8). The vials were assayed for C_2H_2 and C_2H_4 by gas chromatography using a Poropak column (60°C) in a model 5710A Hewlett-Packard gas chromatograph with a flame ionization detector at 145°C. Control vials to test for endogenous C_2H_4 production were always negative, and C_2H_4 production was C_2H_2 dependent. A Hewlett-Packard model 5750 gas chromatograph with a molecular sieve column-5A was used at 65°C with a thermal conductivity detector at 100°C to determine H_2 and monitor O_2 levels (7).

Nitrogenase assays on solid media: To test for acetylene reduction on solid media by single colonies, 3 ml open-ended tubes capped with Suba-seals were inserted over well-grown colonies on BMMSp plates (13). 0.3 ml air was withdrawn and replaced with acetylene. The plates were incubated at 30°C, and periodically gas samples were withdrawn and tested for ethylene production. This ethylene production was always acetylene-dependent.

Nodulation tests: The method of Vincent (9) was used to test for nodulation of clover and lucerne. Clover seeds were surface

sterilized in 5% sodium hypochlorite for 15 min, washed 3 times in sterile distilled water, and allowed to germinate on sterile damp filter paper. Single seedlings were placed on Jensen's medium Petri dishes, which were incubated vertically. This method allowed a similar extent of growth to agar slopes in tubes, with the advantages of being simpler to set up and allowing up to 6 plants per plate (depending on the species used). To test for nodulation of the other legumes used, seeds were surface-sterilized as described above, and were then put in flasks or pots of sterile washed sand. Plants were grown in the glasshouse, and watered with NNB5 medium. Plants were inoculated with the test rhizobia, and some plants were left as un-inoculated controls while others were inoculated with appropriate rhizobia able to nodulate the particular legume. Plants were inspected for nodules after 4 to 8 weeks.

Tests for purity of cultures: All strains were purified before testing for nitrogenase activity by diluting and plating out the culture, picking a single colony, and repeating this procedure at least twice. Single colonies were then picked for further use, and were grown in liquid BMM and streaked on BMM or BMMSp plates for stock cultures. The suspension cultures were tested with bacteriophages (strain T1 and all derivatives are sensitive to phages ϕT10 and Tr8 and bacteriocin T24 (14, 15, 16, 17), and strain L4Sp is sensitive to phage L1 (17)), and were also checked for purity by (a) growth on a range of different solid media which would allow detection of contaminants such as *K. pneumoniae* or *Azotobacter*, (b) morphology and polysaccharide production on solid media, (c) nodulation of the particular legume hosts (red, white and subterranean clover for *R. trifolii*, peas for *R. leguminosarum* and soybeans for *R. japonicum*). On no occasion was there any evidence for contamination of any culture by another bacterium.

All strains were similarly tested after nitrogenase assays, using the cultures from the assay vials. No evidence for contamination was found in any vial.

The T1Sp culture was tested further by first cloning the culture through a white clover nodule (18, 19) after which all the above tests were done as well as inoculating the culture on to other legumes (peas, French beans, soybeans, lucerne, Mung beans, lupins and broad beans) to check for any other contaminating *Rhizobium*. Similarly, after testing for nitrogenase activity, the same series of tests was done on the culture from the assay vial.

RESULTS AND DISCUSSION

As strain T1 is our chosen reference strain for the fast-growing *R. trifolii* (20), the growth characteristics of this strain were investigated on many different media combinations (20). During this investigation, it was found that the spectinomycin-resistant derivative, strain T1Sp, could reduce acetylene at low rates. Other spectinomycin-resistant mutants of strain T1, which were isolated after EMS mutagenesis, were also found to reduce C_2H_2 and evolve H_2 (Table 1). This

TABLE 1

Nitrogenase activity in various spectinomycin-resistant strains of *Rhizobium*

Bacterium	Strain	Acetylene reduction (nmol C_2H_4/hr/mg protein)	Hydrogen production (nmol H_2/hr/mg protein)
R. trifolii	T1	<0.005	<0.01
	T1Sp	0.92	<0.01
	RT52	0.57	1.36
	RT53	1.95	1.36
R. leguminosarum	L4Sp	1.43	2.73
R. japonicum	3I-1b-138	<0.005	<0.01
	RJ1	0.43	6.90

The various *Rhizobium* strains were grown in BMM and diluted with NNB5 medium with succinate (20 mM) as the added carbon source. C_2H_4 production was C_2H_4 dependent and no C_2H_2 reduction or H_2 production appeared in the uninoculated controls.

relationship between the resistance of *Rhizobium* to the antibiotic spectinomycin and the presence of nitrogenase activity was similarly observed for mutants of another fast-grower, *R. leguminosarum*, and the slow-grower *R. japonicum*. No C_2H_2 reduction or H_2 production was detected by the spectinomycin-sensitive *R. trifolii* strain T1 and *R. japonicum* strain 3I-1b-138 under any condition.

The effect of different media and various carbon sources on the detected nitrogenase activity of strain T1Sp was investigated in more detail (Table 2). Acetylene reduction and hydrogen evolution was detected in cultures grown in different media combinations with succinate being the best added carbon source. However, although nitrogenase activity was regularly observed, the actual levels of this activity varied greatly and depended on the growth phase and age of cultures used to inoculate the assay vials, as well as the media used to grow

TABLE 2

The effect of media and carbon sources on nitrogenase activity in *R. trifolii* strain TlSp

Bacterial strain	Growth medium	Dilution medium	Added carbon source	Acetylene reduction[1]	Hydrogen production[2]
TlSp	VYM	NNB5	fructose	46.6	328.9
	VYM	NNB5	ribose	66.2	100.6
	VYM	NNB5	succinate	60.9	230.9
TlSp	V & A	NNB5	fructose	50.7	203.6
	V & A	NNB5	ribose	85.4	191.6
	V & A	NNB5	succinate	90.6	328.4
TlSp	BMM	V & A	glucose	108.6	108.2
	BMM	V & A	succinate	168.9	325.3

1. C_2H_2 reduction is in nmol C_2H_4/hr/mg protein.
2. H_2 production is in nmol H_2/hr/mg protein.
No H_2 or C_2H_4 appeared in uninoculated controls and C_2H_4 production by TlSp was always C_2H_2 dependent.

and dilute cultures. The highest acetylene reducing activities were found when rhizobial cells were first grown with vigorous shaking to stationary phase in BMM and then diluted (at 1:1 ratio) in V & A medium for static culturing in the assay vials with 1% O_2.

The differences in rates of O_2 consumption, H_2 evolution and C_2H_4 production by strain TlSp during static culture in V & A or VYM media is shown in Figure 1. No nitrogenase activity was detected for the first 40 hours of incubation in the assay vials. This period is characterized by a rapid consumption of O_2, and is followed by production of C_2H_4 and H_2. The rates of C_2H_4 and H_2 production increase with further incubation, particularly after the O_2 levels fall below 0.2% (Figure 1). Essentially the same pattern of O_2 consumption, H_2 evolution and C_2H_4 production was found if fructose or ribose replaced succinate as the added carbon source. When acetylene-reducing cultures

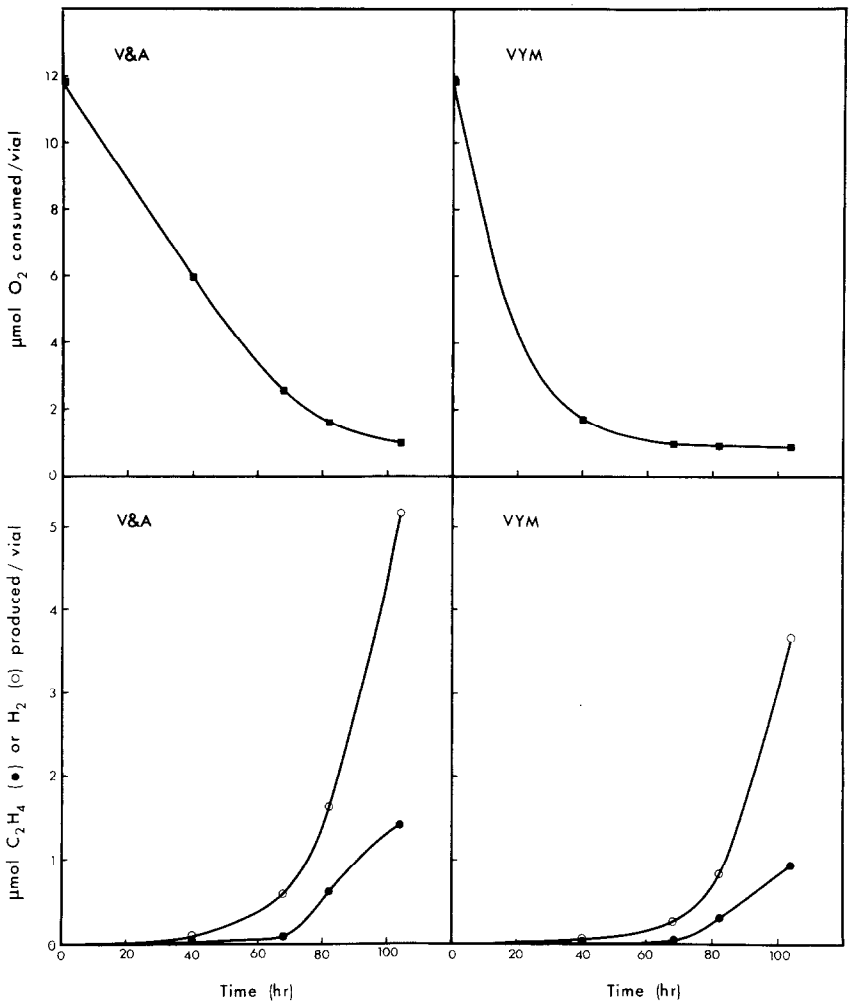

Figure 1. The effect of growth media on O_2 consumption, H_2 evolution and C_2H_4 production by *R. trifolii* strain TlSp.

Strain TlSp was grown either in V & A or VYM media and diluted in NNB5 medium with succinate (20 mM) as the added carbon source. Symbols: ■, O_2 consumption; ○, H_2 evolution; ●, C_2H_4 production.

were examined by phase microscopy, the bacterial cells were found to be rods of about twice the size of aerobically grown (non-fixing) cultures, and very few odd-shaped cells were observed. Presumably the low O_2 concentration is an important trigger in the morphological differentiation of fast-growing rhizobia when they change from bacterium

to bacteroid. This morphological change may also be an important switch for the phenotypic expression of nitrogenase activity both within plant nodules and the assay vials.

Acetylene reduction by strain TlSp was also detected on solid BMM plates containing 100 µg/ml spectinomycin. Single colonies were able to reduce C_2H_2 at a low rate of about 1 nmol C_2H_4/day/colony. Furthermore, single colonies of several spontaneous Sp^R mutants of *R. japonicum* strain 3I-1b-138 on the same medium reduced acetylene at rates of 0.5 to 2 nmol C_2H_4/day/colony.

The fact that individual, well-isolated colonies reduced acetylene shows that it was most unlikely that the cultures contained any contaminants. However, the purity of cultures used in the experiments described in this communication was confirmed with more rigorous controls (described fully in Materials and Methods). On no occasion was there any evidence for contamination by other strains of rhizobia or different bacterial species.

The results in this communication show that there is a correlation in *Rhizobium* between the *in vitro* expression of nitrogenase activity and resistance to the antibiotic spectinomycin. Moreover, this finding enables a repeatable expression of nitrogenase activity, by two different methods, in both fast- and slow-growing rhizobia. This should facilitate the isolation of various classes of mutants of fast-growing rhizobia which are defective in their nitrogen-fixing capacity. The regulation of nitrogen fixation in *Rhizobium* may then be analysed genetically and biochemically.

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