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Siderophore production and utilization by *Rhizobium trifolii*

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Summary. Several strains of *Rhizobium trifolii* were tested for their ability to synthesize and utilize phenolate or hydroxamate types of siderophores. None of the nodulating strains of *R. trifolii* was able to produce detectable amounts of siderophores. Only the non-nodulating strain *R. trifolii* AR6 formed a phenolate siderophore, which stimulated the growth of the siderophore-negative mutant AR65. Other strains of *R. trifolii* could not utilize iron from exogenously supplied Desferal, pseudobactin or citrate. The siderophore from *R. trifolii* AR6 and 2,3-dihydroxybenzoic acid slightly stimulated the growth of some *R. trifolii* strains.

Key words: Siderophore production — Siderophore utilization — *Rhizobium trifolii*

Introduction

Iron is an essential growth factor involved in many metabolic processes in all aerobic organisms. Despite its abundance in the environment, iron is not readily available to bacteria because of its insolubility in neutral pH under aerobic conditions. Many microorganisms produce low-molecular mass compounds termed siderophores, which solubilize ferric iron and facilitate its transport into the cell (Neilands 1981).

The siderophores can be classified chemically into two classes: phenolates and hydroxamates. Fungi generally produce hydroxamate whereas bacteria most often synthesize phenolate siderophores. *Escherichia coli* and *Salmonella typhimurium* produce enterobactin, a cyclic trimer of 2,3-dihydroxybenzoylserine, which is the most powerful ferric-iron-complexing agent known. *E. coli* is able to utilize siderophores produced by other microorganisms, e.g. ferrichrome secreted by fungi (Braun 1985). Iron can be also transported with the aid of citrate.

Each iron transport system in *E. coli* requires an outer-membrane receptor protein and additional functions localized in the cytoplasmic membrane. Expression of all genes responsible for iron transport depends on an additional genetic system (Hantke 1981; Bagg and Neilands 1987).

Rhizobium symbiotically associates with leguminous plants and induces the differentiation of new plant organs, the nodules. Inside the nodules the bacteria reduce molecular nitrogen into ammonium which is made available to the plant host as a nitrogen source.

So far rhizobia have received little attention with respect to iron transport. A catechol-like siderophore was found in *Rhizobium* cowpea (Modi et al. 1985). Smith et al. (1985) described a novel type of siderophore produced by *R. meliloti*. Recently, a catechol-like siderophore has been isolated from non-nodulating strain of *R. trifolii*. This siderophore contains 2,3-dihydroxybenzoic acid and threonine (Skorupska et al. 1988).

In the present study the ability of *R. trifolii* strains to produce and utilize siderophores was examined. Unlike most aerobic bacteria, nodule-forming *R. trifolii* strains do not produce siderophores and they cannot utilize the siderophores produced by other organisms.

Materials and methods

Bacterial strains. A list of strains used is given in Table 1.

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Table 1. Bacterial strains

Strains	Phenotype	Source or reference
Rhizobium trifolii		
24	Nod^+Fix^+	IUNG, Puławy
ST65	Nod^+Fix^+	
ST1-1	Nod^+Fix^+	field isolates from
C5	Nod^+Fix^+	our collection
AR5	Nod^+Fix^+	Deryło et al. (1986)
AR16	Fix^-Muc^-	Deryło et al. (1986)
AR20	Fix^-Muc^-	Deryło et al. (1986)
AR6	Nod^- derivative of AR5	Deryło et al. (1986)
AR65	Nod^+Fix^+ derivative of AR6	this work
2407	Nod^- derivative of 24 cured of pSym	Deryło et al. (1986)
ANU 843	Nod^+Fix^+	B. Rolfe
Salmonella typhimurium LT2	enterobactin producing strain	B. A. D. Stocker
Pseudomonas putida	pseudobactin producing strain	A. N. Pieriebitiuk

Abbreviations: Nod, nodulation; Fix, nitrogen fixation; Muc, mucoid growth; pSym, symbiotic plasmid

Media. Yeast extract/mannitol medium (79CA) was used as complete medium for *Rhizobium* strains (Vincent 1970), M1 medium (NH_4Cl 1 g, K_2HPO_4 2 g, KH_2PO_4 0.5 g, NaCl 0.1 g, glycerol 10 g, H_2O 1000 ml, pH 7.4) was used as minimal, iron-low medium.

Chemicals. 2,3-Dihydroxybenzoic acid and 2,5-dihydroxybenzoic acid were purchased from Sigma, desferrioxamine B mesylate (Desferal) was from Ciba-Geigy. Siderophore *R. trifolii* was obtained according to the method of Young and Gibson (1978); pseudobactin was isolated as described Meyer and Abdallah (1978).

Assay for presence of siderophores. R. trifolii strains were incubated overnight in 79CA then diluted tenfold in 100 ml 79CA with 100 μM 2,2′-bipyridyl or M1 supplemented with 0.5% casamino acid. After 48 h, supernatants of the cultures were extracted twice with ethyl acetate. Extracts were dried and the pellets were dissolved in 1 ml ethanol. The Arnow reaction was used to assay the phenolic type of siderophores (Arnow 1937). Siderophores of *R. trifolii* AR6 and *S. typhimurium* LT2 were used as positive controls.

The production of hydroxamate. This was tested by adding 5 mM $FeCl_3$ to 1 ml of the supernatants of the cultures. Additionally, all cultures were tested by the Csaky reaction (Csaky 1948). In the Csaky reaction, hydroxylamine was used as a positive probe.

Effect of chelators on growth of R. trifolii *strains.* 5 ml of M1 medium supplemented with thiamin 2 μg/ml, biotin 2 μg/ml and appropriate compounds was inoculated with 0.5 ml of an overnight culture of *Rhizobium* strains in 79CA medium. Chelators were used in following concentrations: 2,3-dihydroxybenzoic acid 50 μM, siderophore AR6 2 μg/ml, pseudobactin 10 μM, Desferal 10 μM, sodium citrate 10 mM, supernatant of the culture of *R. trifolii* AR6 0.2 vol. $FeCl_3$ (10 μM) was added to each medium with the chelator. The initial absorbance was approximately 0.02 at 560 nm. The cultures were grown for 48 h at 28° C with shaking and then turbidity was measured at 560 nm.

Thin-layer chromatography. Ethyl acetate extract of *R. trifolii* AR5 culture dissolved in ethanol was tested by thin-layer chromatography on silica gel SiG0 F254 (Merck) in different solvent systems. Two parallel plates were used for each solvent system. After development of the chromatograms, one plate was sprayed with Hathway reagent (0.1 M $FeCl_3$ in 0.1 M HCl) added to an equal volume of 0.1 M potassium ferricyanide (Hathway 1969) and the second one was treated with 0.1 M $FeCl_3$ in 0.1 M HCl for testing iron-binding activity.

Results

Siderophore assays

Wild-type strains of *Rhizobium trifolii* and non-fixing (Fix^-) and non-nodulating (Nod^-) mutants were tested for siderophore production. Only the strain *R. trifolii* AR6 grew on minimal medium without added iron. All the other strains of *R. trifolii* (Table 2) did not grow in M1 medium without added iron.

To test the possibility of siderophore production these strains were incubated in M1 medium with casamino acid or in 79CA medium with 2,2′-bipyridyl. Supernatants from cultures of all tested strains gave negative results when assayed for the presence of hydroxamates by the method of Csaky (1948). On the other hand, most of the ethyl acetate extracts of *R. trifolii* cultures produced a weak blue reaction with the Hathway reagent (Hathway 1969) used for detection of phenolates (Table 2). Usually the synthesis of all types of siderophores is repressed by addition of iron, but the production of the substance responsible

Table 2. Production of substances giving Arnow-positive reaction

Strains	Production of catechol in medium (μg/ml)	
	M1	79CA
R. trifolii		
24	0.04	0.02
AR5	0.65	0.0
ST65	0.13	0.0
ST1-1	0.02	1.1
A29	0.19	0.46
C5	0.22	2.0
ANU843	0.19	0.2
AR16	0.13	2.2
AR20	0.7	0.0
AR6	7.0	4.4
2407	0.2	0.1
S. typhimurium LT2	10.0	n.t.

Production of catechol is given in μg/ml of DHBA equivalent
Medium M1 was supplemented with 0.5% casamino acid; medium 79CA was supplemented with 100 μM 2,2'-bipyridyl. n.t. = not tested

for the blue reaction with the Hathway reagent was not affected by addition of 10 μM $FeCl_3$ to the growth medium.

To characterize this substance which gave a blue reaction, ethyl acetate extract of *R. trifolii* AR5 culture in 79CA medium was chromatographed on silica gel in three solvent systems together with different phenolates (Table 3). On developed plates we found a spot which gave a blue colour with the Hathway reagent. However, this spot was not identical to that produced by standard substances. When the plates were sprayed

Table 3. Chromatographic properties of ethyl acetate extract of *R. trifolii* AR5 culture

Compound	R_F value in solvent system		
	I	II	III
Extract AR5	0.71	0.62	0.54
2,3-DHBA	0.70	0.47	0.40
2,5-DHBA	0.50	0.42	0.37
Salicylic acid	n.t.	n.t.	0.63
Extract AR6	0.6	0.11, 0.16	n.t.
Extract *S. typhimurium*	0.64, 0.47 0.29	0.03, 0.05 0.09	n.t.

Solvent systems: (I) butanol acetic acid water (40:10:10); (II) benzene methanol acetic acid (45:8:4); (III) benzene. n.t. = not tested

with 0.1 M ferric chloride in 0.1 M HCl, the extract of AR5 showed no reaction, whereas the spots of siderophores AR6 and *Salmonella typhimurium* LT2 gave a red colour.

We conclude that *R. trifolii* wild-type strains synthesize small amounts of an unknown phenolic substance(s) without iron-binding activity and consequently without function in iron transport. These results do not completely disprove siderophore production by the nodulating *R. trifolii* strains but indicate that these bacteria do not readily secrete detectable typical phenolate or hydroxamate siderophores.

Chelator utilization

To discover whether some chelating substances could improve the growth of rhizobia, the follow-

Table 4. Effect of chelators on growth of *R. trifolii* strains

Addition to medium M1′	Absorbance at 560 nm of *R. trifolii* cultures							
	24	AR5	ANU843	AR16	AR20	2407	AR6	AR65
None	0.07	0.08	0.10	0.13	0.13	0.08	0.60	0.10
$FeCl_3$	0.13	0.19	0.16	0.16	0.18	0.11	0.72	0.23
$FeCl_3$, 2,3-DHBA	0.11	0.15	0.17	0.16	0.26	0.14	0.80	0.36
$FeCl_3$, siderophore AR6	0.10	0.19	0.16	0.22	0.16	0.12	0.85	0.27
$FeCl_3$, pseudobactin	0.10	0.10	0.13	0.16	0.19	0.11	0.78	0.19
$FeCl_3$, citrate	0.02	0.05	0.05	0.03	0.05	0.07	0.86	0.16
$FeCl_3$, Desferal	0.02	0.09	0.07	0.11	0.07	0.05	0.76	0.16
$FeCl_3$, supernatant AR6	0.15	0.25	0.18	0.21	0.26	0.23	—	0.31
0.2% yeast extract + 0.5% casamino acid	0.22	0.30	0.42	0.38	0.43	0.32	0.82	0.40

Mean values from at least three samples are given. For details see Materials and methods. 2,3-DHBA = 2,3-dihydroxybenzoic acid

ing substances were used: desferrioxamine B (Desferal) the catechol-like siderophore from *R. trifolii* AR6, the supernatant from culture of AR6, 2,3-dihydroxybenzoic acid, (2,3-DHBA), pseudobactin from *Pseudomonas putida* and citrate. All the chelators were used in the presence of $FeCl_3$. Only *R. trifolii* AR6, which produces a siderophore, grew well on all the media used in this experiment (Table 4). The growth of other strains depended on iron: in the presence of $FeCl_3$, the growth was slightly stimulated by 2,3-DHBA and the siderophore of *R. trifolii* AR6.

The effect of the stimulation was more weakly expressed on minimal medium without added iron (data not shown). The growth of AR65 was most actively stimulated by 2,3-DHBA. We assume therefore, that this strain is defective in synthesis of 2,3-DHBA, the precursor of rhizobial AR6 siderophore (Skorupska et al. 1988). The elimination of the symbiotic plasmid from *R. trifolii* 24 did not change the ability to utilize the ferric chelators.

Discussion

Rhizobia as aerophilic bacteria possess an iron-dependent respiratory type of metabolism and require iron for their growth. It was thought that in iron deficiency rhizobia, like many other aerobic bacteria, might secrete siderophores. However, Smith et al. (1985) tested several wild-type *R. meliloti* strains and they found only one which produced a novel type of siderophore. Also Rioux et al. (1986) screened *R. leguminosarum* for production of hydroxamate or phenolate siderophores and they obtained negative results.

The results of our study showed that most *R. trifolii* strains did not synthesize the common types of siderophores and they were not able to utilize iron from different siderophores such as Desferal and pseudobactin.

Other compounds containing carboxylic groups can mobilize iron and transport it into cell, e.g. anthranilic acid (Rioux et al. 1986), citric acid (Roessler and Nadler 1982). In our study citrate did not stimulate the growth of *R. trifolii* in minimal medium.

Some aerobic bacterial species have been described which lack a high-affinity iron transport system. *Yersinia enterocolitica* is unable to produce siderophores but can utilize siderophores of other bacteria (Robins-Brown and Prpic 1985). *Legionella* cannot produce or utilize any siderophores (Reeves et al. 1983). Morse et al. (1987) found that under iron-limitation conditions *Neisseria* species synthesize a 36–37-kDa protein which may play a role in iron acquisition.

The free-living rhizobia probably take up iron by a low-affinity iron transport system and in the symbiotic state they acquire iron by means of a plant host.

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