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# Siderophore activity in *Rhizobium* species isolated from different legumes

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***Rhizobium*** **strains isolated from nodules of the different legumes including wild-growing plants were examined for their siderophore activity. Fifteen of the 84 screened rhizobial strains were able to grow under conditions of limited iron supply. Nine of them gave orange halos in the assay with Chrom azurol S. Among these strains were *Rhizobium* sp. (*Ononis*) and *Rhizobium* (*Genista*), producing hydroxamates and phenolates. These compounds could promote the growth of siderophore-negative bacteria on iron-deficient media. The results imply that the hydroxamates from G1 and O1 strains may belong to the monohydroxamate class of siderophores.**

Iron is an element essential for almost all living organisms. Although iron is abundant in nature its availability for microorganisms is limited by poor solubility at neutral pH under aerobic conditions [1, 2]. At low iron ion concentration bacteria and fungi produce chelating compounds, called siderophores [1, 2]. Most siderophores belong to the hydroxamate or catechol classes. Recently the assay with Chrom azurol S (CAS)[1] has been introduce to detect of siderophores, irrespective of their chemical structure [3]. The presence of an iron chelator (siderophore) is indicated by a change in the CAS medium color from blue to orange.

Bacteria of the genera *Rhizobium, Bradyrhizobium* and *Azorhizobium* (rhizobia) are able to fix nitrogen within the nodules formed on the roots or stems of legume plants. The effective nodules produce a variety of iron-containing proteins i.e. nitrogenase, leghemoglobin, ferredoxin. Surprisingly only few *Rhizobium* or *Bradyrhizobium* strains have been found to produce a siderophore of any type. So far, all the tested strains were isolated from cultivated plants [3-6]. In our comparative study bacteria isolated from nodules of wild-growing plants were screened for siderophore production.

## MATERIALS AND METHODS

***Bacteria.*** *Rhizobium leguminosarum* biovar *trifolii* 24 (wild type) and *R.l.* bv. *trifolii* AR6, a siderophore producing strain, were described earlier [6]. The remaining *R. leguminosarum* bv. *trifolii* strains were isolated by E. Kowalczuk, *R. leguminosarum* bv. *phaseoli*, *R. meliloti*, *Bradyrhizobium* sp. (*Lupinus*) by R. Staniewski. Rhizobia from wild-growing plants were kindly provided by B. Różyńska.

***Media.*** Rhizobia were routinely grown on the mannitol-yeast extract medium, 79 CA [7]. Minimal M9 [8] medium contained: thiamine, pantothenic acid and biotin each at a concentration of 2 μg/ml.

[1] Abbreviations: CAS, Chrom azurol S; DHBA, 2,3-dihydroxybenzoic acid; EDDA, ethylenediamide di(*o*-hydroxyphenylacetic acid); HDTMA, hexadecyl- trimethylammonium bromide.

***Chemicals.*** Chrom azurol S (CAS) was purchased from Serva, etylenediamine di(*o*-hydroxyphenylacetic acid) (EDDA), 2,2'-dipyridyl, 1,10-phenanthroline from Sigma and desferrioxamine B (Desferal) from Ciba-Geigy.

***Siderophore assays.*** Hydroxamates were tested by the method of Csaky [9] using the modified hydrolysis procedure of Gibson & Magrath [10], with hydroxylamine as a standard. Catechol contents were estimated spectrophotometrically at 310 nm and after extraction to ethyl acetate with Hathway reagent as described earlier [6]. 2,3-Dihydroxybenzoic acid (DHBA) was used as a positive control. Chrom azurol S – hexadecyltrimethylammonium bromide (HDTMA) was used for detection of all types of siderophores. CAS agar was prepared as described by Schwyn & Neilands [3] with some modifications. Because HDTMA is toxic to some rhizobia, therefore bacteria were grown on an iron low medium (M9 or 79 CA) for 48 h, then were killed with chloroform and the CAS solution containing 0.7% of agar was poured onto the plates. Orange halos surrounding the colonies indicated siderophore production. CAS solution was also used for quantitation of siderophore production in the culture supernatants, ethyl acetate extracts and in solutions of partially purified siderophores.

Strains producing the known siderophores: pseudobactin (*Pseudomonas* sp. AP267) and enterobactin (*Salmonella typhimurium*) served as positive controls.

***Purification of siderophores.*** Exopolysaccharides from the spent growth medium were removed by precipitation with 3 vol. of ethanol. Then, the supernatants of the *Rhizobium* sp. (*Ononis*) and *Rhizobium* sp. (*Genista*) strains were dried and the residues were dissolved in deionized water. Samples (5 ml) of the materials were passed through a Sephadex G-25 column using water-methanol (10:1, v/v) as a solvent. Fractions of 5 ml were collected and tested with CAS solution. The CAS positive fractions were pooled, lyophilized and resuspended in water. Approximately 20 mg of partially purified siderophore was obtained from 1 liter of the culture supernatant.

***Bioassay for siderophore.*** Iron-limiting media were routinely obtained by supplementing the 79 CA or M9 media with 100 μM of EDDA. The strains which were unable to produce siderophores were seeded on the plates and sterile filter paper disks impregnated with siderophore were placed. The bacteria were incubated at 28°C for 2–4 days and by that time growth around the disks could be visible.

## RESULTS AND DISCUSSION

Eighty-four strains isolated from nodules of cultivated (*Trifolium, Phaseolus, Pisum, Medicago* and *Lupinus*) and wild-growing legume plants (*Astragalus, Cytisus, Genista, Ononis, Termopsis* and *Desmodium*) were screened for siderophore production. Fifteen of the strains were able to grow on M9 medium or 79 CA with 50 μM EDDA, which indicates that they had a high affinity system of iron uptake. The production of extracellular siderophore was then tested by using the CAS reagent as described by Schwyn & Neilands [3]. Nine isolates gave positive reactions with CAS, changing the color of CAS medium from blue to orange (Table 1). None of six *R. meliloti* strains growing on M9 gave a positive reaction with CAS. Barton *et al.* [11] who examined various *R. meliloti* strains found that these bacteria produced a constitutive ferric reductase, what suggested that this enzyme participates in iron assimilation.

Significant correlation between the ability to grow on iron-low medium and CAS reactivity was found in the case of the strains isolated from *Trifolium, Astragalus, Genista* and *Ononis*. All bacteria producing orange halos on CAS agar were less sensitive to the iron-chelators than CAS-negative rhizobia (Table 2). The results indicate that the rhizobia form iron-chelators (presumably siderophore) of higher affinity to iron than the affinity of EDDA, 2,2-dipyridyl or 1,10-phenanthroline. Recent studies indicate that the majority of rhizobia are unable to form any siderophores. For example, Schwyn & Neilands [3] out of 13 strains of *R. meliloti* screened, found only one that produced rhizobactin, a novel type of siderophore. Similarly, only one of 20 of *Bradyrhizobium japonicum* strains synthesises an iron-chelating compound that was identified as citric acid [5]. In the present work we have tested bacteria isolated from nodules of wild growing legume plants. About 10% of them secreted chelators, probably siderophores as proved by growth on CAS agar.

Table 1
*Growth on low-iron medium and siderophore production by rhizobia*

| Species of *Rhizobium* | Number of isolates | | |
|---|---|---|---|
| | Total | Growing on M1 | CAS positive* |
| *R. l. trifolii* | 30 | 2 | 2 |
| *R. l. phaseoli* | 6 | 0 | 0 |
| *R. l. viciae* | 6 | 0 | 0 |
| *R. meliloti* | 8 | 0 | 0 |
| *Bradyrhizobium* sp. (*Lupinus*) | 6 | 0 | 0 |
| *Rhizobium* sp. (*Cytisus*) | 5 | 0 | 0 |
| *Rhizobium* sp. (*Astragalus*) | 5 | 2 | 2 |
| *Rhizobium* sp. (*Ononis*) | 5 | 2 | 2 |
| *Rhizobium* sp. (*Termopsis*) | 5 | 1 | 0 |
| *Rhizobium* sp. (*Genista*) | 5 | 2 | 2 |
| *Rhizobium* sp. (*Desmodium*) | 5 | 0 | 0 |

*CAS positive, forming orange halo around colonies.

The bacteria isolated from *Genista* (strain G1) and from *Ononis* (strain O1) were examined in more detail. The culture supernatants of O1 and G1 strains gave positive reactions for hydroxamates and phenolates. Simultaneous production of the phenolate and hydroxamate siderophores has been reported in the case of *Azotobacter, Enterobacteriacae* and *Pseudomonas* [12-14].

Phenolates were extracted from spent growth medium with ethyl acetate and the ethyl acetate and aqueous fractions were assayed for CAS activity. Both fractions gave a positive reaction with CAS. The ethyl acetate extracts from G1 and O1 strains were used in a bioassay with the *Escherichia coli aro*B mutant. This strain is deficient in enterobactin production and can grow on iron-deficient media only in the presence of exogenous siderophores. We have found that the ethyl acetate extracts were able to promote growth of *E. coli aro*B.

The aqueous fractions were purified on Sephadex G-25 column, eluted with $H_2O$-methanol (10:1, v/v) and fractions were assayed for CAS activity. The elution profiles of the aqueous fractions from G1 and O1 strains (Fig. 1) were almost identical (Fig. 1) (the second one is not shown). The CAS positive fractions were examined for the presence of phenolates and hydroxamates. The level of hydroxamates was correlated with CAS activity. The relative molecular mass of the compounds was estimated by

Table 2
*Growth of Rhizobium strains on 79 CA medium with chelators*
EDDA, ethylenediamine di(*o*-hydroxyphenylacetic acid); DP, 2,2-dipyridyl; PHT, 1,10-phenanthroline.

| Species of *Rhizobium* | EDDA | DP | PHT |
|---|---|---|---|
| | 50 μM | 100 μM | 100 μM |
| *R. l. trifolii* 24 | – | – | – |
| *R. l. trifolii* 93 | – | – | – |
| *R. l. trifolii* AR6 CAS* | + | + | + |
| *R. l. trifolii* 288 CAS* | + | – | + |
| *Rhizobium* sp. (*Ononis*) CAS* | + | + | + |
| *Rhizobium* sp. (*Genista*) CAS* | + | + | + |

*CAS positive, see Table 1.

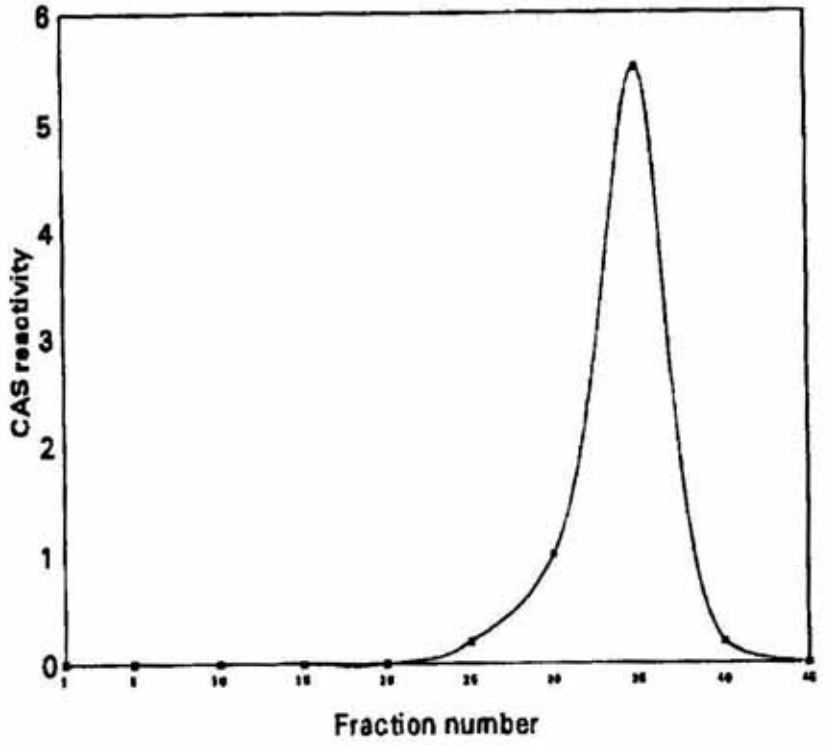


*Fig. 1. Chromatographic profile of culture supernatant of Rhizobium G1 strain on Sephadex G-25 column. The material was eluted with water-methanol (10:1, v/v). Fractions (5 ml) were collected and assayed for siderophore by the colorimetric reaction with CAS. CAS reactivity was shown in arbitrary units.*

gel filtration as about 200, i.e. below these of trihydroxamates (about 500–1000). Most of the hydroxamate siderophores have three hydroxamic chelating groups [12], but some organisms produce mono- and dihydroxamates. Jalal *et al.* [15] presented evidence that fungi had active uptake systems to assimilate iron from mono- and dihydroxamates. The bioassay applied showed that hydroxamates from G1 and O1 strains were able to promote the growth of the siderophore-negative rhizobia that were not stimulated by trihydroxamates such as desferrioxamine B (Desferal) or pseudobactin (Table 3).

Transport of the iron-siderophore complexes into bacteria requires outer membrane proteins that serve as specific receptors. For example, *Morganella morganii* does not recognize trihydroxamates but responds to siderophores of different structure [16].

Table 3

*Promotion of growth of bacteria on iron-deficient media by different siderophores*

Bacteria were plated on 79 CA medium supplied with: 50 μM EDDA in the case of *R. trifolii* 24, 100 μM EDDA for *R. trifolii* 288 and *E. coli aro*B. No growth (–), zone (diameter) of stimulation: 10–12 mm (+), 12–15 mm (++), 15–20 mm (+++), inhibition of growth — antibiotic activity (a); NT, not tested.

| Iron chelators | Strains | | | |
|---|---|---|---|---|
| | *R. trifolii* 24 | *R. trifolii* 288 | *R. meliloti* L5-30 | *E. coli aro*B |
| Desferal | – | + | a | NT |
| Pseudobactin | – | + | a | NT |
| G1 hydroxamate | + | +++ | a | + |
| O2 hydroxamate | + | +++ | a | + |

The chemical properties of isolated compounds together with the results of the bioassay suggest that these hydroxamates can be classified as monohydroxamates. There are no reports concerning hydroxamate production by rhizobia, but it is known that rhizobia can utilize this type of siderophores [17, 18].

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