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SIDEROPHORE CONTAINING 2,3-DIHYDROXYBENZOIC ACID AND THREONINE FORMED BY *RHIZOBIUM TRIFOLLI**

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An iron-binding compound was isolated from ethyl acetate extract of culture supernatant fluid of *Rhizobium trifolii* AR6 and was purified by iron-exchange chromatography. The compound was characterized by UV and IR. It contained 2,3-dihydroxy-benzoic acid and threonine and was accumulated during stationary phase of growth in iron-deficient media. Synthesis of the siderophore was repressed by $FeCl_3$. In iron limited medium the compound promoted growth of *R. trifolii* strains.

Iron is an essential growth factor for nearly all bacteria but its availability for microorganisms is limited by its poor solubility at neutral pH. Bacteria are able to transport iron into the cell in several ways [1, 2]. Under limited iron conditions the bacteria produce and release low molecular weight compounds termed siderophores. The siderophores solubilize iron and facilitate its transport into the cell. Siderophores are classified chemically into two main classes: phenolates and hydroxamates. The best studied phenolate siderophore is enterobactin, identified as the cyclic trimer of 2,3-dihydroxybenzoylserine [3, 4, 5, 6]. Catechol-like siderophores are commonly produced by bacteria. The siderophore production in the genus *Rhizobium* has been recently reported [7, 8]. Siderophore of *Rhizobium* cowpea consists of 2,3--dihydroxy-benzoic acid (DHBA) threonine and glycine [7]. Smith *et al.* [8] described structurally novel type of siderophore from *R. meliloti* DM4. This siderophore, designated rhizobactin, contained the ethylenediamine group which is exceptional in a natural product. So far there are no reports

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on siderophores in *R. trifolii*. In this study the catechol-like siderophore from *R. trifolii* AR6 was isolated and characterized.

MATERIALS AND METHODS

Bacterial strains. R. trifolii AR6 non-nodulating (Nod⁻) mutant was used as a source of siderophore. Wild type *R. trifolii* 24 was used for testing the biological activity of the siderophore.

Media. R. trifolii strains were routinely grown on mannitol-yeast extract agar, 79CA [9]. The mineral salts medium (M1) used contained per liter: NH_4Cl, 1 g; K_2HPO_4, 2 g; KH_2PO_4, 0.5 g; NaCl, 0.1 g; mannitol, 10 g; pH 7.4.

Isolation and purification of siderophore from R. trifolii. R. trifolii strain AR6 was grown in 3 l volume of M1 medium in 5 l flask at 28°C, 48 h, with aeration. The bacteria were removed by centrifugation, the supernatant fluid was collected and supplemented with $FeCl_3$ (0.2 mM). The development of mauve colour was due to the presence of ferric-siderophore. The next steps of purification of the siderophore were carried out according to Young & Gibson [10]. The supernatant was passed through a DEAE-cellulose column (3 cm diameter, 19 cm length). The column was washed with 0.5 l of sodium phosphate buffer 0.01 M, (pH 7) and eluted with 2 M NH_4Cl (pH 7). Fractions which gave mauve colour with 0.2 mM $FeCl_3$ were pooled, the pH adjusted to 1 with concentrated H_2SO_4 and extracted three times with 1 volume of ethyl acetate. The extracts were combined and washed with equal volume of sodium phosphate buffer (0.01 M, pH 7). Then ethyl acetate extract was dried over anhydrous Na_2SO_4 and concentrated by rotary evaporation.

Siderophore assays. Bacteria grown in M1 medium at 28 °C, were centrifuged from 20 ml of the culture and the supernatant fluid after acidification was extracted three times with equal volume of ethyl acetate as described above. The siderophore concentration was determined in ethanol solutions with the Hathway reagent (0.1 M $FeCl_3$ in 0.1 N HCl added to equal volume of 0.1 M potassium ferricyanide) at 700 nm [11, 12]. The amount of siderophore was determined as amount of catechol [7]. There was a linear correlation between A_{700} and the concentration of catechol at the range 0 - 20 μg of catechol/ml. Before estimation, the siderophore was diluted in ethanol.

Hydroxamates were tested by adding 50 μM $FeCl_3$ to 1 ml of supernatants and by the method of Csáky [13] using the modified hydrolysis procedure of Gibson & Magrath [14].

Thin-layer chromatography. The siderophore dissolved in methanol was checked for purity on thin-layer plates of silica gel Si60 F254 (Merck) in following solvent systems: A, chloroform-ethanol (4:1); B, isopropanol - 25% NH_3 aq. - water (100:10:10); C, butanol-acetic acid-water (12:3:3). After development of the chromatogram, the plate was checked under UV light and a test strip was sprayed with iron reagent (0.1 M $FeCl_3$ in 0.1 N HCl).

The spots showing light-blue fluorescence under UV light and yielding a mauve colour with iron reagent were identified as catechol-like compound.

Assay of iron-binding by siderophore was conducted by mixing 10 μg of purified siderophore in ethanol with 0.1 μCi $^{55}FeCl_3$ (specific activity 130 MBq/mg Fe, Swierk, Poland) followed by thin-layer chromatography on silica gel in solvent B. The plates were exposed to Rentgen-XS films (13 × × 18 cm) for 2 days followed by spraying with iron reagent.

Spectroscopic procedures. Absorption spectra in the visible and UV ranges were obtained with a model 635 Varian Techtron UV-VIS Spectrophotometer. Infrared spectra were obtained with a Specord 70 using KBr discs.

Identification of amino acids in Rhizobium siderophore. 2.0 mg of rhizobial siderophore isolated from the supernatant of *R. trifolii* AR6 were hydrolyzed with 6 N HCl at 100°C during 6 h under nitrogen. The hydrolysate was then dried in the stream of nitrogen and the residue was redissolved in water and analyzed using Aminochrom II amino acid analyzer, paper and thin-layer chromatography. For paper chromatography Whatman paper No 1 was used with a solvent system containing n-butanol-pryridine-water (1:1:1, by vol.) or phenol-water (4:1, w/w). Thin-layer chromatography was performed on the plates with silica gel Si60 F254 (Merck) in the solvent system containing n-propanol-water (7:3) or on the plates with cellulose Merck with two solvent systems containing n-butanol-acetic acid-water (60:15:15) or n--propanol - 0.2 M NH_3 aq. (3:1). The chromatograms were checked under UV light 350 nm and developed with the ninhydrin reagent.

Identification of 2,3-dihydroxybenzoic acid (2,3 DHBA) in siderophore. Thin-layer plates of silica gel TLC Si60 F254 Merck were used with a solvent system containing benzene-methanol-acetic acid (45:8:4). The hydrolysate of siderophore was spotted along with standards: 2,4 DHBA; 2,3 DHBA and 3,4 DHBA. The chromatograms were checked under UV light fluorescence and developed by spraying with 1% $FeSO_4$ or iron reagent.

Bioassay of Rhizobium siderophore. The ability of siderophore to support the growth of *R. trifolii* 24 has been tested on M1 iron-free medium. For this purpose sterile filter paper disks, impregnated with 5 μg of siderophore have been placed on agar plates inoculated with 10^5 of *Rhizobium* cells. The plates were examined after 2 - 4 days for zones of growth around the disks.

RESULTS

Siderophore synthesis. Ten wild type strains as well as ten non-nodulating (Nod) and non-fixing (Fix) mutants of *R. trifolii* were checked for production of both hydroxamate and catechol type siderophores. Supernatant fluids from cultures of all tested strains gave negative results when assayed for the presence of hydroxamates by the method of Csáky [13]. On the other hand, most of ethyl acetate extracts of *R. trifolii* cultures produced

a weak blue reaction with the Hathway reagent [12] specific for phenolate siderophores. However, the production of the substance responsible for blue reaction with Hathway reagent was not repressed by addition of 10 μM $FeCl_3$ to growth medium. The supernatant fluids from culture of *R. trifolii* AR6, 24SM and 24GR gave strong positive reaction when assayed for catechol-type siderophores with Hathway reagent [11, 12]. These strains were derived from wild type *R. trifolii* 24 as non-nodulating mutants. The synthesis of catechol-like compound was suppressed by addition of 6 μM $FeCl_3$ to growth media. The synthesis of both types of siderophores is repressed by the addition of iron into-culture medium [2]. *R. trifolii* AR6 strain was chosen to further study for isolation and characterization of the siderophore. Synthesis of a catechol-like substance by *R. trifolii* AR6 was measured during growth in M1 medium without iron added (Fig. 1). The siderophore was found to accumulate slowly in the culture supernatant and maximum synthesis was noted in the stationary phase of growth. Synthesis

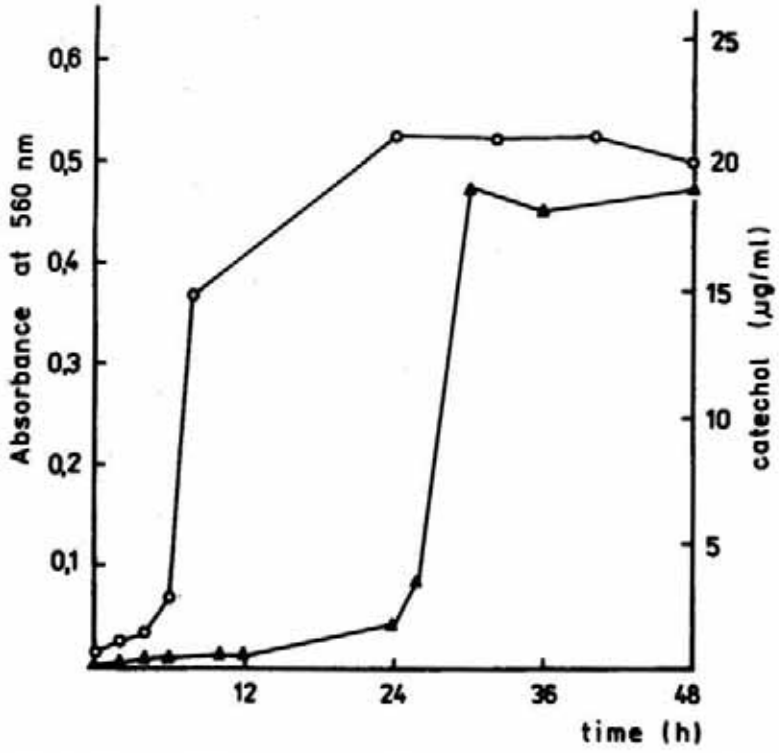

Fig. 1. Synthesis of catechol-like substance during growth of *R. trifolii* AR6. Bacteria were inoculated into 400 ml of M1 medium and samples of 20 ml were removed at intervals for absorbance measurement (at 560 nm) of growth (○) and concentration of siderophore (as catechol) in the culture supernatant (▲).

of this compound was repressed by added iron and 6 μM $FeCl_3$ completely blocked the production of siderophore (Fig. 2).

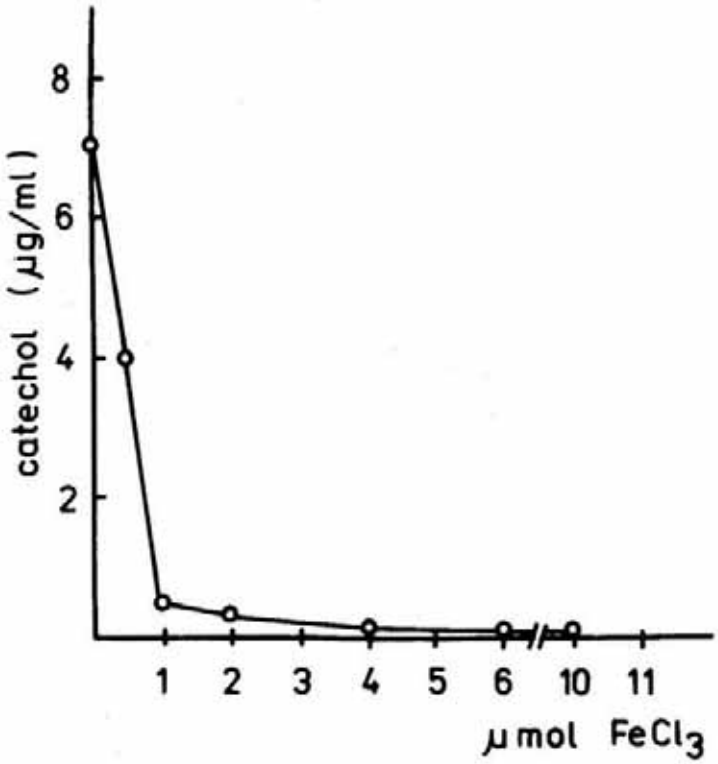

Fig. 2. Effect of iron concentration on the production of siderophore by *R. trifolii* AR6 growing in M1 medium. The siderophore concentration was estimated as described in Fig. 1.

Biological activity of siderophore. To study biological activity of rhizobial siderophore the strain *R. trifolii* 24 was used. This strain did not grow on M1 iron-free medium. However, the zone of the growth appeared around the discs impregnated with siderophore of *R. trifolii* AR6 (Plate 1).

Siderophore characterization. Ethyl acetate extract from 3 l of 2 day culture of *R. trifolii* AR6 yielded 18 mg of catechol-like compound. Iron-free form of siderophore was soluble in methanol, ethanol, ethyl acetate and poorly soluble in water, chloroform and carbon tetrachloride. Ferric-siderophore was better resolved in water. The purified siderophore (Materials and Methods) was chromatographed on silica gel plates with different solvent systems. Thin-layer chromatography of the purified siderophore yielded spots with the following R_f values: solvent A, 0.08; solvent B, 0.69; solvent C, 0.8. The spots gave mauve reactions with iron reagent, blue reactions with

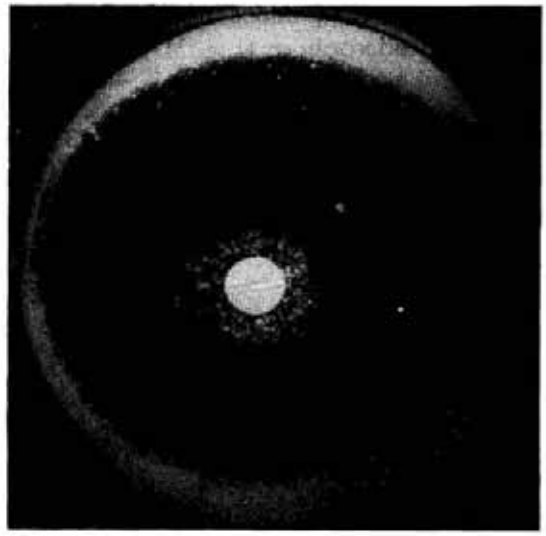

Plate 1. Growth promoting activity of rhizobial siderophore exerted on *R. trifolii* 24 strain.

Hathway reagent and fluoresced under UV light. Ethyl acetate extracts from 400 ml of the supernatant of three wild type strains *R. trifolii* AR5, 24, ANU843 chromatographed on silica gel did not give the spots corresponding to rhizobial siderophore.

Methanol solution containing purified siderophore displayed an absorption spectrum at pH 7 with maxima at 252 and 314 nm and at pH 12 with maxima at 253 and 342 nm (Fig. 3). This characteristic shift in absorption maxima when the pH was adjusted to values between 7 and 12 pointed to phenolic character of this compound. Ferric-siderophore in methanol solution (pH 7) showed two absorption maxima slightly moved towards the longer wavelengths (259 and 325 nm) (Fig. 4). Thin-layer chromatography of hydrolyzed siderophore of *R. trifolii* AR6 with standards: 2,4 DHBA; 2,3 DHBA and 3,4 DHBA showed the presence of catechol that ran identically to 2,3 DHBA. Positive ninhydrin reaction was not observed unless siderophore was hydrolyzed. To identify amino acid content the hydrolysate of catechol--type siderophore from *R. trifolii* AR6 was subjected to paper and thin layer chromatography (Materials and Methods). The presence of threonine was confirmed by using amino acid analyzer.

The infrared spectrum of purified siderophore resembled to some extent that of catechol (Fig. 5). There was a broad band near 3560 cm^{-1} in the region of phenolic OH stretching. The absorption at 1465 cm^{-1} as well as at 740 and 780 cm^{-1} may tentatively be attributed to C-H vibration of benzene ring. The two latter bands are presumably connected with the substitution of benzene at positions 1, 2, 3. The IR spectrum contained

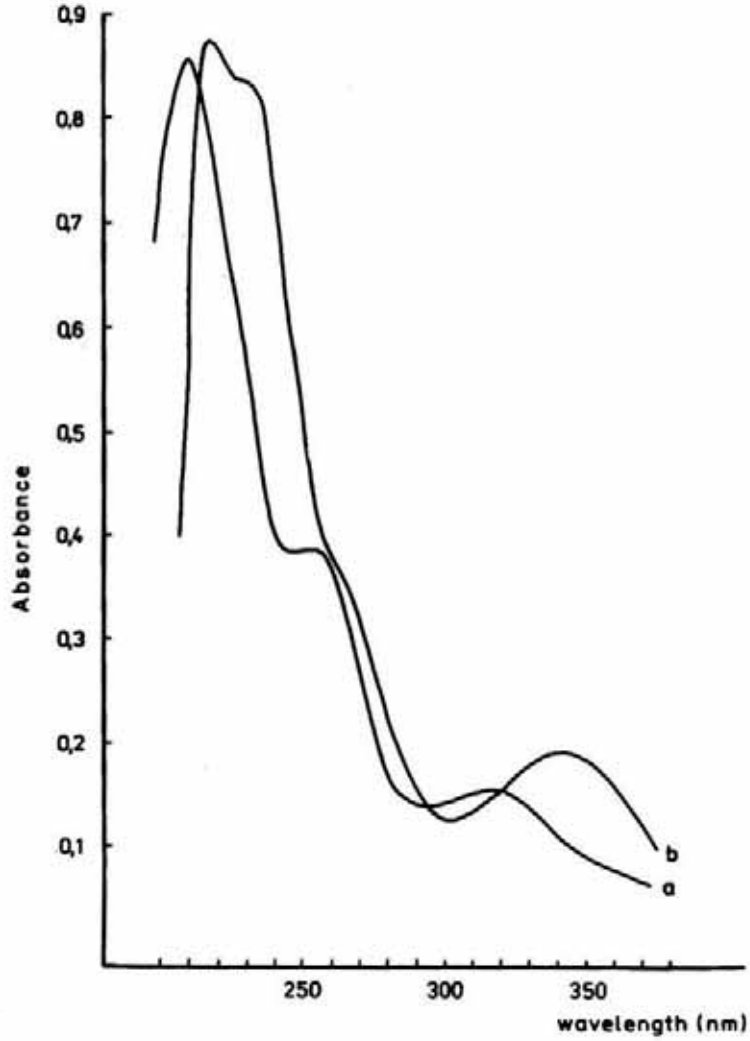

Fig. 3. UV absorption spectra of purified iron-free rhizobial siderophore at pH 7 (a) and pH 12 (b) in methanol-water (9:1) solution.

intensive band at 1633 cm^{-1}, typical for an amide C=O stretch. The broad band near 3360 cm^{-1} may include also absorption due to the presence of N-H group. Moreover, spectrum contained peaks at 1331, 1161 and 1036 cm^{-1} which may correspond to phenolic OH and to alkyl OH vibrations, respectively. Bands near 2920 and 2850 cm^{-1} may be ascribed to presence of alkyl moiety in siderophore. All bands were broadened due to hydrogen bonds inter- and intramolecular.

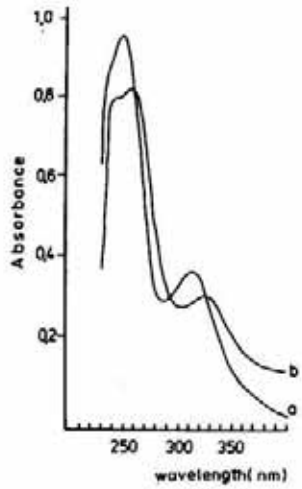

Fig. 4. UV absorption spectra of purified siderophore a, in iron-free form; b, and of its ferric-chelate obtained by addition 1 mmol $FeCl_3$ in methanol. The samples were obtained by dissolving 50 µl of DMSO solution of siderophore (15 mg/ml) in 2 ml of methanol.

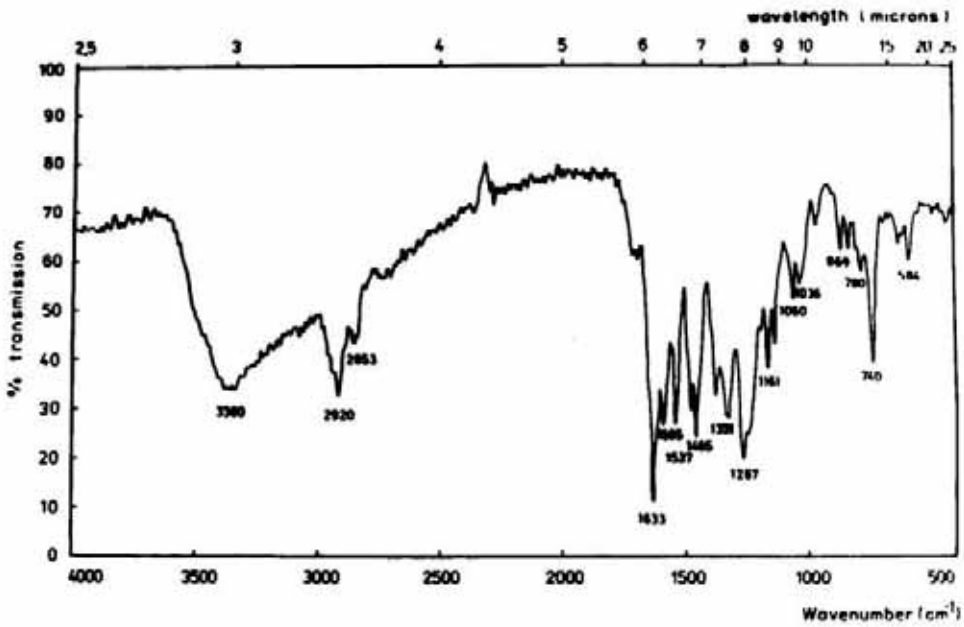

Fig. 5. Infrared spectrum of rhizobial siderophore in KBr disc.

Proof of iron binding character. Iron binding by catechol-like compound was demonstrated by thin-layer chromatography on silica gel. ^{55}Fe ions alone did not migrate in solvent B system. After mixing with siderophore, $^{55}Fe^{++}$ migrated together with siderophore, what was demonstrated by autoradiography technique (Plate 2).

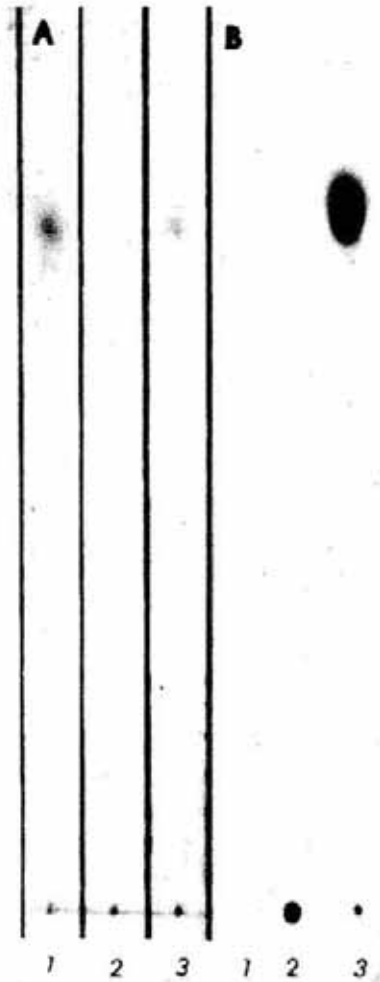

Plate 2. Iron-binding ability of siderophore of *R. trifolii* AR6. Thin-layer chromatography on A, silica-gel; and B, autoradiogram; of 1. ferric-siderophore; 2. $^{55}FeCl_3$; and 3. ^{55}Fe--siderophore.

DISCUSSION

The mechanism by which *rhizobia* acquire iron is poorly understood at present. Siderophores, generally produced by aerobes and facultative anaerobes, exceptionally were detected in rhizobia [7, 8]. It has been suggested that these microbial symbionts are forced to acquire iron from plant host [8]. Testing numerous wild type and symbiotic mutants of *R. trifolii* we found siderophore activity only in some Nod$^-$ mutants. Similarly to other described siderophores [15, 16] synthesis of the rhizobial siderophore was repressed by the presence of iron in growth media. In low-iron media *R. trifolii* AR6 produced 6 mg of this substance per liter. The low concentration was typical for phenolate whereas hydroxamate siderophores were produced in much higher amounts. Kinetics of the synthesis of the siderophore was similar to those observed in the case of other catechol-like siderophores [7, 17].

A number of lines of evidence point to the presence of phenolic group in rhizobial siderophore. The absorbance in the UV and fluorescence of the molecule indicate the presence of an aromatic ring. Moreover, the changes in UV absorbance at pH between 7 and 12 are characteristic for phenolates. The IR spectrum indicates that benzene ring was substituted in 1, 2, 3 positions. Finally catechol was identified as 2,3-dihydroxybenzoic acid (DHBA) by co-chromatography with authentic DHBA. Additionally to DHBA, threonine was chromatographically detected as a component of the siderophore. A number of described siderophores contained the serine or threonine linked by ester bonds e.g. the enterobactin contained three ester-linked serine residues (2). The absence of absorption near 1.760 cm^{-1} (IR spectrum) points to the lack of the ester bonds in rhizobial siderophore. These results indicate that rhizobial siderophore is not a structural analogue of the enterobactin. A catechol type siderophore containing threonine 2,3-dihydrobenzoic acid and spermidine was found in *Agrobacterium tumefaciens*, the bacterium related to *Rhizobium* [18]. This siderophore named agrobactin has analogous structure to parabactin in which OH-group of central catechol in position 3 is lacking [19]. Both compounds contain the threonyl residue in the form of oxazoline ring. Exposure to acid leads to opening of the ring and destroys the biological activity of the siderophores. The siderophore of *R. trifolii* isolated in acidic conditions preserved its ability to stimulate growth of bacteria on the iron low media. It may suggest that the rhizobial siderophore is not identical with agrobactin or parabactin.

The structure of the rhizobial siderophore has not been yet elucidated but its iron-binding activity has been verified. The purified siderophore not only binds iron but also promotes the growth of *R. trifolii* strains on iron deficient medium. The biological activity allows to define the described substance as a siderophore.

Siderophores are generally formed by pathogenic microorganisms but production of agrobactin is not correlated with the virulence [20]. Siderophore of *R. trifolii* similarly to agrobactin may be involved in iron assimilation *ex planta*, as it is produced by the strains which lost their symbiotic properties.

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