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Isolation and Partial Characterization of Catechol-Type Siderophore from *Pseudomonas stutzeri* RC 7

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Abstract. *Pseudomonas stutzeri* RC 7 grown under iron-deficient conditions produced catechol-type siderophore, which was identified to be arginine conjugate of 2,3-dihydroxy-benzoic acid. Hydroxamic acids were not detected. The concentration of siderophore in the culture supernatant was maximal after 24 h of growth. Addition of iron to the medium increased bacterial growth but repressed the production of siderophore.

Many bacteria derepress high-affinity iron transport system when starved of iron [14]. These systems consist of an iron chelator or siderophore, which is either hydroxamate or catechol compound; expression of both types is regulated by iron [15]. Many Pseudomonads are reported to produce both types of siderophores [4, 7, 12]. Fluorescent pigment-like pyoverdine produced by Pseudomonads is also reported to act as siderophore [11]. Among non-fluorescent Pseudomonads, *Pseudomonas stutzeri* has been reported to produce nocardamine (Ferrioxamine E) as siderophore [10]. These compounds have a high affinity for Fe^{3+}, and a number of them participate in the transport of iron into the cells [13]. *Pseudomonas stutzeri* RC 7 produced catechol-type siderophore under iron-depleted conditions. This siderophore also possessed two essential features shown by other Pseudomonads [7, 10, 12]; it is produced only under iron-deficient conditions and secondly, it forms a very stable complex with Fe^{3+}. The present paper describes the isolation and partial characterization of the catechol-type siderophore from *Pseudomonas stutzeri* RC 7. It also deals with the evidence, which shows the biphasic relationship between the iron content of the medium and the siderophore produced by the organism.

Materials and Methods

Culture and growth conditions. *Pseudomonas stutzeri* RC 7 was isolated in our laboratory and was identified with the help of CABI Mycological Institute (Ferry Lane, Surrey TW93AF, UK). A small inoculum of the culture was added to succinate medium (5 ml in a test tube, 15 × 150 mm) containing (g/L): succinic acid, 4.0; $(NH_4)_2SO_4$, 1.0; KH_2PO_4, 3.0; K_2HPO_4, 0.1; and $MgSO_4 \cdot 7\ H_2O$, 0.2. The pH was adjusted to 7.0. This was incubated on a rotary shaker (200 rpm) for 12 h. This 12-h-old culture was subsequently used as inoculum in all the experiments. *Pseudomonas stutzeri* RC 7 was grown at 25°C in 250-ml Erlenmeyer flasks containing 100 ml succinate medium and subjected to mechanical agitation. Growth was measured turbidometrically at 600 nm, and cell dry weights were calculated with the appropriate calibration curve.

Isolation, estimation, and characterization of siderophore. Catechol-type siderophore was extracted in ethyl acetate from the culture supernatant of *Pseudomonas stutzeri* RC 7 after 24 h of growth. The amount of catechol-type siderophore was measured in ethyl acetate extract by Arnow's method [1]. Gibson and Magrath's method [5] was used for the hydroxamate estimation. The ethyl acetate extracts were concentrated, and the residues were dissolved in solvent ether. Catechol-type siderophore was identified by TLC on silica gel G, with benzene/toluene/acetic acid (2 : 2 : 1 by vol.) as a solvent system and with authentic catechols. The band corresponding to authentic 2,3-DHBA was scraped out and isolated by preparative TLC and the same solvent system. The identification of the compound was further confirmed by ultraviolet spectroscopy. For the amino acid detection, the purified siderophore was acid hydrolyzed; the amino acids were separated and identified by paper chromatography with the solvent system butanol/acetic acid/water, 12 : 3 : 5 by vol. The chromatograms were developed with ninhydrin reagent (0.2% wt/vol in acetone) [18].

Results

Pseudomonas stutzeri RC 7 was isolated in our laboratory and was identified with the help of CABI Mycological Institute (UK). *Pseudomonas stutzeri* RC 7, when grown under agitated conditions (200 rpm) at 25°C, produced a very high amount of

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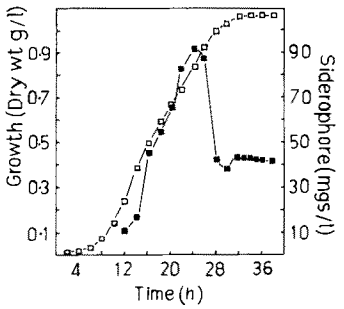

Fig. 1. Siderophore production (■) at different stages during the growth (□) of *Pseudomonas stutzeri* RC 7.

Table 1. Identification of siderophore by thin-layer chromatography

Standard DHBAs	R_f values
2,3-DHBA	0.32
3,4-DHBA	0.1
3,5-DHBA	0.04
Salicylic acid	0.58
Hydrolysate of siderophore	0.31
Unhydrolyzed siderophore	0.32

siderophore. Addition of 1 m*M* $FeCl_3$ to the culture supernatant developed wine color, suggesting the presence of catechol-type siderophore, which was further confirmed by Arnow's method [1]. Absence of hydroxamic acids was confirmed by Gibson and Magrath's method [5]. Colorimetrically detectable concentration of the siderophore appeared in the culture supernatant after 12 h of growth, reaching its maximum at 24 h of growth when grown under agitated conditions at 25°C (Fig. 1). The decrease in the siderophore activity was observed as the organism entered into its stationary phase; this is unusual and could be owing to the degradation of siderophore.

The presence of 2,3-dihydroxy benzoic acid (2,3-DHBA) in the ethyl acetate extract was confirmed by TLC with authentic catechols as standards (Table 1). This was purified by preparative TLC. The ultraviolet (UV) spectrum of the purified siderophore was found to be identical with that of the authentic 2,3-DHBA (Fig. 2). Catechol-type siderophores are generally conjugates of amino acids [16]. We detected the presence of arginine as an amino acid in the acid hydrolysate of the siderophore with paper chromatography (Table 2).

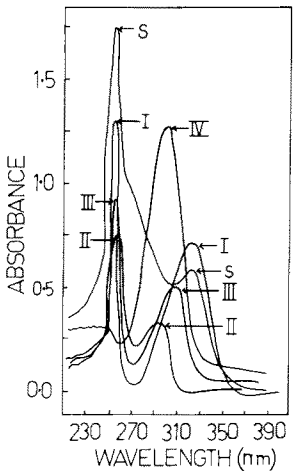

Fig. 2. Ultraviolet spectra of the purified siderophore (S) and authentic catechols 2,3-DHBA (I); 3,4-DHBA (II); 3,5-DHBA (III); and salicylate (IV).

Table 2. Identification of amino acids by paper chromatography

Amino acids	R_f values
Threonine	0.5
Glycine	0.48
Aspartate	0.46
Arginine	0.41
Ornithine	0.31
Phenylalanine	0.78
Tryptophan	0.27
Lysine	0.37
Siderophore hydrolysate	0.41

Addition of Fe^{3+} to the culture medium (600 μg/L) before inoculation increased the growth but decreased the siderophore production. Further increase in the Fe^{3+} concentration totally inhibited siderophore production without affecting the growth of *Pseudomonas stutzeri* RC 7 (Fig. 3). *Pseudomonas stutzeri* RC 7 when grown on 8-hydroxyquinoline-treated medium showed less siderophore production than when grown on untreated medium; this suggests that the concentration of iron present as contaminant was optimum for the production.

Discussion

The fluorescent pseudomonads are reported to produce a catechol-type siderophore [12]. In the case of nonfluorescent pseudomonads, *Pseudomonas*

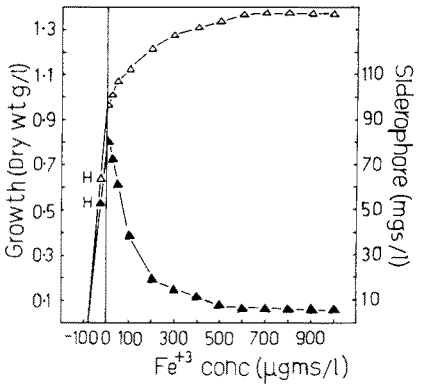

Fig. 3. Siderophore (▲) and growth (△) of *Pseudomonas stutzeri* RC 7 as a function of Fe^{3+} present in the succinate medium. Point H corresponds to the standard medium pretreated with hydroxyquinoline. Negative value on the abscissa corresponds to the amount of Fe^{3+} in the medium as a contaminant.

stutzeri (ATCC 17588) is reported to produce nocardamine, which acts as a siderophore [10]. *Pseudomonas stutzeri* RC 7 produced a catechol-type siderophore under iron-deficient conditions. The production of siderophore reached its maximum at 24 h of growth. The siderophore produced by *Pseudomonas stutzeri* RC 7 was found to be a derivative of 2,3-DHBA. Catechol-type siderophores are generally conjugates of amino acids and 2,3-DHBA [16]. The role of amino acids in the siderophore complex is unclear, although Knosp et al. [6] have reported that unconjugated DHBA does not promote iron uptake in *Azotobacter vinelandii*. We detected the presence of arginine in conjugation with the siderophore after acid hydrolysis.

Iron (Fe^{3+}) was found to be the regulatory factor for the siderophore production by *Pseudomonas stutzeri* RC 7. The biphasic relationship (Fig. 3) between the production of siderophore and the concentration of iron was observed. Similar observations were made for the production of nocardamine in *Pseudomonas stutzeri* [10] as well as in other organisms [2, 17]. According to Fig. 3, when the iron was removed from the succinate medium with 8-hydroxyquinoline before innoculation, the siderophore production was found to be less than when grown on untreated succinate medium. This suggests that the concentration of iron present as contaminant was optimum for the maximum siderophore production and that up to this concentration the production is iron (Fe^{3+}) dependent. This indicates that the first step in the catechol-type siderophore synthesis requires the presence of iron. This seems to be the case in the biosynthesis of enterochelin, which facilitates the uptake of iron in *Escherichia coli* [16]. The first enzyme involved in the biosynthesis of the aromatic moiety of enterochelin, 3-deoxy-D-arabinoheptulosonate-7-phosphate synthase, has been reported recently to be an iron protein [8]. The specific activity of this enzyme as a function of iron shows a biphasic relationship [9]. Addition of iron to the medium (600 µg/L) totally inhibited siderophore production but showed maximum growth. This indicates that the biosynthesis of catechol-type siderophore in *Pseudomonas stutzeri* RC 7 is repressed by excess iron (>200 µg/L Fe^{3+}).

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