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AN ANTAGONISTIC ACTIVITY OF *ESCHERICHIA COLI* AGAINST A SPECIES OF *PSEUDOMONAS*[1,2]

TAN-CHI HUANG and MIAU-CHAN CHANG

Institute of Botany, Academia Sinica, Nankang, Taipei Taiwan, Republic of China

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Abstract

An endophytic *Pseudomonas* sp. was found to be sensitive to *E. coli*. The antagonistic phenomenon is different from those caused by colicins, coliformin or antifungal compound of *E. coli*. The antagonistic activity of *E. coli* against the isolate is specific. Nitrate and sucrose, trehalose, or glucose have to be present in the assay medium for having the inhibitory activity. The optimal pH for the activity was at 7.0 to 8.0. The optimal temperature was at 30°C. The mechanism of inhibition was due to the inhibitory activity of nitrite accumulated by *E. coli*.

Introduction

The ecological relationship of *E. coli* with other organisms is one of the important subjects and has been studied by micorbiologists. It is known that colicins are produced by some strains of *E. coli* against some species of *Salmonella* and *Shigella*, and inhibit the growth of other strains of *E. coli* (Ryan *et al.*, 1955; Goebel *et al.*, 1955; Fredericq, 1956). In addition to colicins, strains of *E. coli* which possess antifungal activity have been reported (Freyschuss *et al.*, 1950; Maritin, 1953).

During our studies on the interaction between *E. coli* and other microorganisms, we found that an endophytic *Pseudomonas* sp. isolated from the tissue of rice plant is sensitive to *E. coli*. Further experiments revealed that the inhibition phenomenon of our finding was different from reported antagonism caused by *E. coli*. Specificity of the antagonism, factors affecting the antagonistic activity, and the mechanism causing the inhibition were studied.

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Materials and Methods

Source of microorganisms

The *Pseudomonas* sp. which is sensitive to *E. coli* was isolated from tissues of rice according to procedures described by Huang and Chang (1976). *E. coli* NIHJ, *Salmonella paratyphi* B, *S. swiseptifer* 2276, *S. typhosa, Serratia marcescens, Shigella* sp., *Klebsiella* sp., *Hafnia* sp., *Proteus* sp., *Mycobacterium phlei* and *M. pseudotuberculosis* were obtained from C. W. Ho, College of medicine, National Taiwan University; *E. coli* B7A, an enterotoxigenic strain, was obtained from Dr. P. Echeverria, NAMRU II Unit of Taipei; *E. coli* B, *E. coli* C-51, *E. coli* C-57, *E. coli* ML-30, *E. coli* ML-30A and *E. coli* K-12 were obtained from Dr. Y. H. Tseng, Department of Botany, National Chung-Hsing University; Strains of *Erwinia* were obtained from Dr. W. D. Douglas, Department of Scientific and Industrial Research, New Zealand; *Enterobacter aerogenes, Sarcina lutea, Pseudomonas tolaasi, P. syringae, P. solanaceanum, Agrobacterium tumefaciens, Rhizobium phaseoli,* strains of *Xanthomonas, Staphylococcus, Streptococcus, Bacillus* and strains of *E. coli* from pigs, chicken and fishes were isolated in this laboratory.

Medium

The medium containing 0.5% peptone, 1.5% sucrose, 0.2% Na_2HPO_4, and 0.05% $Ca(NO_3)_2$ was routinely used for the antagonistic activity assay.

Detection of the antagonistic activity

Following assays were used for detecting the antagonistic activity:

1. Direct contact method: The coli-sensitive *Pseudomonas* sp. was plated on the petri dish by double layer method. A loop of *E. coli* or other testing bacteria taken from slant was transferred to the center of the plate and incubated at 30°C. Inhibition zone was examined after 24 hr of incubation.

2. Cellophane cup method: The cellophane cup was made with a small glass column (0.6 cm in length and 1.0 cm in diameter). One end of the column was wrapped by cellophane paper forming a cup-shape coutainer. In this cup, 0.5 ml of melted agar medium was filled and was solidified. Then the *E. coli* or other bacteria was transferred on the surface of the medium by a loop. This cup was then placed on the plate which contained coli-sensitive *Pseudomonas* sp.

3. Paper disc method: Paper disc method was used when the inhibitory activity of the filtrate of bacterial culture was examined. Ten μl of filtrate was added to each disc with a micropipete.

Cultures of the *Pseudomonas* sp. at log phase were used in the above assays. Old cultures affected the sensitivity of assay.

Isolation of mutants

Mutants of *E. coli* unable to reduce nitrate to nitrite were induced and isolated according to the method described by Jose' Ruiz-Herrera *et al.* (1969).

Nitrite determination

To determine nitrite, 0.1 ml of 0.8% sulfanilic acid in 0.86 M sulfuric acid was added to 2 ml of nitrite containing sample, mixed well, and then 0.1 ml of 0.5% naphthylamine in 0.15 M sulfuric acid was added and mixed. After 5 minutes, the absorbancy of the mixture was read at 540 nm with a Gilford spectrophotometer.

*Sensitivity of the **Pseudomonas** sp. to nitrite*

The sensitivity of the *Pseudomonas* sp. to nitrite was assayed by agar plate method. Small amount of the culture at log phase were evenly spread on the surface of the agar medium (10^4 cells/plate) which contained nitrite ranged from zero to 5000 ppm. The plates were incubated at 30°C for 18 hr and then the bacteria in plate were suspended and collected in 5 ml of distilled water. The O. D. of bacterial solution collected from each plate was then measured.

Purification of the inhibitory compound

E. coli culture inoculated in liquid medium was used for the isolation of the inhibitory compound. The culture was incubated anaerobicly for 18 hr in liquid medium at 30°C. The cells were removed by centrifugation and the suppernatant was concentrated by rotatory evaporation to ten times. Active carbon was added to the concentrated filtrate to have a final concentration of 1% (by wt.) and allowed to react for a half hour. The active carbon was then removed by filtrating through filter paper. The filtrate was then concentrated further for 5 times and then put on a DEAE-cellulose column (40 cm×6 cm) equilibrium with 0.01 M Tris buffer, pH 8.0. The inhibitory compound was then eluted with 0.1 N NaCl in 0.01 M Tris buffer. The inhibitory active fractions were concentrated and further purified by passing through sephadex G-10 column (50 cm×6 cm). Fractions which have the inhibitory activity were collected and their properties were studied.

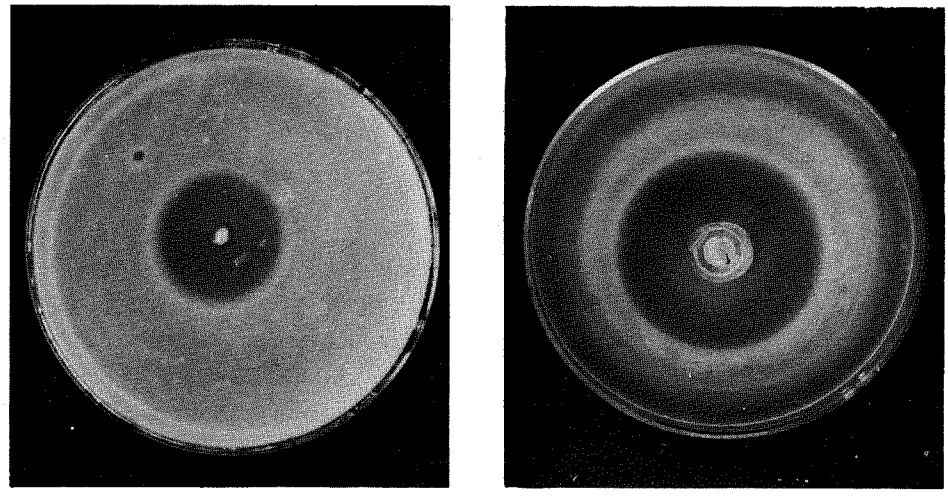
Results

Detection of the antagonistic activity

As shown in Fig. 1a, in the direct method, the inhibition zone was observed

after 12 hr of incubation. The zone reached its maximum size after 24 hr. The zone gradually reduced and finally disappeared within 48 hr due to the re-growth of the *Pseudomonas* sp.

The inhibition zone was also demonstrated by cellophane cup method. As shown in Fig. 1b, the inhibition zone was very clear after 24 hr of incubation. With this method, there is no directed contact between the *Pseudomonas* sp. and *E. coli*, so the inhibitory compound must have diffused through the cellophane paper and affected the *Pseudomonas* sp..



(1a) (1b)

Fig. 1. Inhibition zone formed by ***E. coli*** against the coli-sensitive *Pseudomonas* sp. (1a) By direct contact method; (1b) By cellophane cup method.

No inhibition zone was observed when the filtrate, without concentration, of *E. coli's* liquid culture was assayed by paper disc method. However, after the filtrate was concentrated to five times, clear inhibition zone could be detected by paper disc method. The filtrate of anaerobic culture had higher activity than the aerobic culture. The inhibitory compound in filtrate was concentrated by evaporation. Certain inactive compounds were removed from the filtrate by active carbon. The inhibitory compound was then purified by DEAE-cellulose ion exchange and Sephadex gel filtration. The inhibitory active fractions pooled from Sephadex column showed positive in nitrite test and had an absorption spectrum identical to authetic nitrite (Fig. 2).

Specificity of the antagonistic activity

One hundred and seventy three bacterial strains were tested for their inhibitory activity against the *Pseudomonas* sp. by direct contact method. As

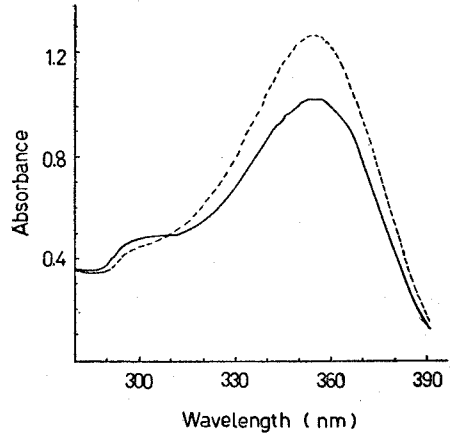


Fig. 2. Absorption spectra of the purified inhibitory compound and the authetic nitrite.
----: authetic nitrite; —: inhibitory compound

Table 1. *Inhibitory activity of bacterial strains against the coli-sensitive* ***Pseudomonas*** *sp.*

Organism	Number of strains tested	Number of the active strains
E. coli (human origin)	35	35
E. coli (pig origin)	20	20
E. coli (chicken origin)	20	20
E. coli (fish origin)	20	20
Salmonella spp.	3	3
Shigella sp.	1	1
Proteus sp.	1	1
Hafnia sp.	1	1
Klebsiella sp.	1	1
Enterobacteria aerogenes	1	1
Serratia marcescens	1	1
Erwinia spp.	16	6
Pseudomonas spp.	3	0
Xanthomonas spp.	7	0
Rhizobium phaseoli	1	0
Agrobacterium tumefaciens	1	0
Sarcina lutea	1	0
Micrococcus luteus	1	0
Staphylococcus spp.	5	0
Streptococcus spp.	10	0
Corynebacterium michiganense	1	0
Mycobacterium spp.	2	0
Bacillus spp.	21	0

shown in Table 1, all strains of *E. coli* and related enteric bacteria have a positive reaction. The others are negative, except *Erwinia*, a genus taxonomically belongs to Enterobacteriaceae, which have both positive and negative strains.

In addition, 109 bacterial strains were tested for their sensitivity to *E. coli* by the same method. As shown in Table 2, only the newly isolated *Pseudomonas* sp. is sensitive to *E. coli*.

Table 2. *Sensitivity of bacterial strains to E. coli.*

Organism	Number of strains tested	Number of sensitive strain
Pseudomonas sp. (the new isolate)(1)	1	1
Pseudomonas tolaasi	1	0
Pseudomas solanaceanum	1	0
Pseudomonas syringae	1	0
Agrobacterium tumefaciens	1	0
Azotobacter vinelandii	1	0
Azotobacter chroococcum	1	0
Beijerickia mobile	1	0
Sarcina lutea	1	0
Micrococcus lutea	1	0
Staphylococcus spp.	5	0
Streptococcus spp.	10	0
Xanthomonas spp.	7	0
Erwinia spp.	16	0
Bacillus spp.	21	0
Bacterial strains isolated from rice plant(2)	40	0

(1) This is the coli-sensitiv *Pseudomonas* sp. used in this study.
(2) These bacterial strains were isolated from rice in this laboratory.

Factors affecting the antagonistic activity

Media with different nitrogen sources were checked for their effects on the activity. Results revealed that the presence of nitrate in the assay medium was required for producing the inhibitory zone. Optimum concentration of nitrate for the activity was found to be in the range of 0.0021 to 0.0042 M. In addition to nitrate, it was found that one of some carbohydrates also has to be present in the medium simultaneously. Such carbohydrates including sucrose, lactose, trehalose, ducitol, mannitol, mannose, fructose, sorbitol, inositol, glucose, galactose, xylose, arabinose, glyceraldehyde, dihydroxyacetone, and pyruvate, in a concentration of 1%, were individually tested for their effects on the activity. It was found that trehalose, sucrose, or

glucose had to be present in the assay medium. The others did not have significant effect on the antagonistic activity. Ions such as Na^+, K^+, Mg^{++}, Mn^{++}, Ca^{++}, Cl^-, SO_4^{--} did not have effect on the activity.

When assayed with the direct contact method and cellophane paper method, the optimal pH for the inhibitory activity was found to be between 7.0 and 8.0. The optimal temperature was found to be 30°C.

Nitrate reduction and the inhibitory activity

All the bacterial strains which showed inhibitory activity in this study have positive nitrate reduction activity. Mutants of *E. coli* lacking this activity lost the inhibitory activity coincidentally. However, not all bacterial strains with positive nitrate reduction activity detected by broth method under oxygen limiting condition (Salle, 1954) have the inhibitory activity. For example, *Bacillus subtilis, B. cereus, Staphylococcus aereus* and some species of *Erwinia* tested in this study are positive in their nitrate reduction activity when assayed by broth method, but negative in the inhibitory activity.

Expression of nitrate reductase is dependent on environmental conditions (Showe and DeMoss, 1968). In this study, agar plate under aerobic condition was used as the assy condition, and there were bacterial interaction between the *Pseudomonas* and the tested bacteria involved during the assay. It was found that *Bacillus subtilis, B. cereus,* and *S. aereus* were not able to reduce nitrate although their nitrate reductase are positive when assayed by broth method. Strains which had inhibitory activity were found to be able to reduce and accumulate nitrite in agar plate in the direct contact or cellophane cup method.

Sensitivity of the ***Pseudomonas*** *sp. to nitrite*

Authentic nitrite was used to test the sensitivity of the isolate to nitrite. As shown in Fig. 3 and 4, the sensitivity was affected by certain environmental factors, especially the kinds of carbohydrates present and the pH of the medium. Various carbohydrates were tested for their effects on the sensitivity of the *Pseudomonas* sp., it is found that the sensitivity of the *Pseudomonas* sp. to nitrite was enhanced about hundred times in the presence of trehalose, sucrose or glucose, which are the same kinds of carbohydrates required for the antagonistic activity of *E. coli* against the *Pseudomonas* sp..

Discussion

Certain kinds of inhibitory activity of *E. coli* against microorganisms have

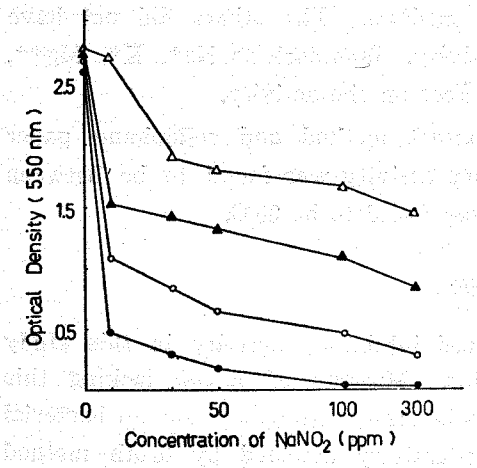


Fig. 3. Effect of sodium nitrite concentration at different pH on the growth of the *Pseudomonas* sp.. Sucrose was added in the assay medium at a concentration of 1%.
●-●: pH 5.0; ○-○: pH 6.0;
▲-▲: pH 7.0; △-△: pH 8.0

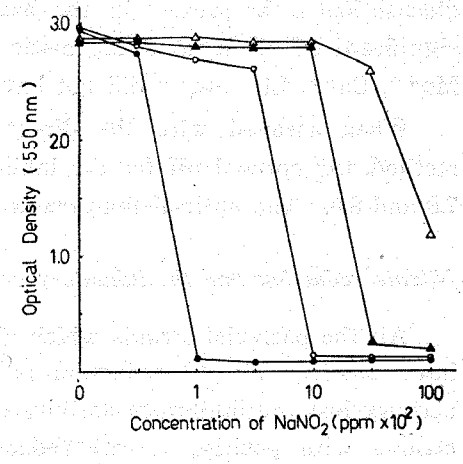


Fig. 4. Effect of sodium nitrite concentration at different pH on the growth of the Pseudomonas sp.. There was no sucrose in the assay medium.
●-●: pH 5.0; ○-○; pH 6.0;
▲-▲: pH 7.0; △-△: pH 8.0

been reported. Among them, producing colicins are phenomenon having been tentatively studied (Nomura, 1967). Colicin is protein in nature and can not diffuse through cellophane paper. The antagonistic activity of *E. coli* against the *Pseudomonas* sp. reported in this study is different from that of colicin or colicinlike compounds, because the inhibitory compound in this case is a small molecule and not protein in nature.

The results of this study indicate that the mechanism of causing inhibition was due to the accumulation of nitrite in the assay plates by *E. coli:* (1) All the active strains showed positive nitrate reductase activity. (2) Mutants of *E. coli* losing the nitrate reductase activity lost the inhibitory activity simultaneously. (3) Nitrate had to be present in the assay medium for producing the inhibition zone. (4) Accumulation of nitrite was demonstrated in agar plate with *E. coli.* (5) The inhibitory compound purified from *E. coli* culture was found to have an absorption spectrum identical to nitrite. (6) The *Pseudomonas* sp. was very sensitive to nitrite when growing in the assay medium. (7) The sensitivity of the *Pseudomonas* sp. to both *E. coli* or nitrite was enhanced by the same kinds of carbohydrates.

The antagonistic activity of *E. coli* against the *Pseudomonas* sp. is specific. Among over one hundred bacterial strains tested, only strains of *E. coli* and enteric bacteria are positive. Since the inhibition was shown to be caused by the accumulation of nitrite in agar plates, it can not exclude the possibility that other untested bacterial strains will also have the same inhibitory activity

if they are able to accumulate nitrite under the assay condition. Nitrate reductase is an inducible enzyme. Its expression is affected by the composition of the medium, the enviromental factors and the methods used in the assay (Payne. 1973). Thus the inhibitory spectra of bacteria obtained from this experiment may be mordified if different assay condition is used.

Acknowledgments

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大腸桿菌對一株假單胞桿菌所形成之拮抗作用研究

黃檀溪 張妙璨

中央研究院植物研究所

自植物體內所分離出來之一株假單胞桿菌受大腸桿菌的抑制。此拮抗現象之形成跟以往所發表如大腸桿菌之產生 Colicins, Coliformin 或其他抗眞菌物質不同。此一拮抗現象具有特異性。又在測定此一現象的培養基中必須要有硝酸和蔗糖，海藻糖（Trehalose）或葡萄糖的存在。形成此拮抗作用之最適酸鹼度是 7.0 到 8.0，最適溫度是攝氏 30 度。實驗結果證明此拮抗現象是由於大腸桿菌將硝酸還原成亞硝酸所造成的結果。