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Growth Inhibition and Pyocin Receptor Properties of Endotoxin from *Pseudomonas aeruginosa* (38023)

JOHN DYKE AND RICHARD S. BERK
Department of Microbiology, Wayne State University, School of Medicine, Detroit, Michigan 48201

The chemical and biological characterization of endotoxin from *Pseudomonas aeruginosa* has come under intensive study within the last few years. Homma *et al.* (1) and Naoi *et al.* (2) were able to extract an endotoxin composed of two components separable by electrophoresis. Component I consisted of a lipopolysaccharide–protein complex, while component II consisted of DNA, RNA, and a polyribose complex. Homma and Suzuki (3) also reported that the isolated protein from *P. aeruginosa* endotoxin exhibited bacteriocinogenic (pyocin) activity. Studies by others (4, 5) indicate that there are several types of pyocin substances. Higerd *et al.* (4) demonstrated that one of these pyocins consisted of a reversibly contractile protein particle resembling *E. coli* T-even phage tails. Adsorption of "relaxed" particles to a sensitive indicator strain of *P. aeruginosa* resulted in contraction of the pyocin particle.

In addition, many strains of *P. aeruginosa* undergo spontaneous lysis (auto-plaques) when cultured on solid media. However, no infectious particle has been isolated from the virus-like plaques. Lysis can be stimulated by several antibacterial substances (6). Current studies indicate that endotoxin from *P. aeruginosa* concomitantly exhibits bacteriocinogenic activity as well as enhances auto-plaque formation. Because of the apparent relationship between endotoxin, pyocin activity, and the auto-plaque phenomenon, the purpose of this paper is to further describe some of these biological phenomena in *P. aeruginosa.*

Materials and Methods. Organisms. *Pseudomonas aeruginosa* strain W_9 served as a sensitive indicator strain for the titration of pyocin produced by strain C_9. Pyocin was purified and titrated as previously described (7).

Medium. The composition of the growth medium for pyocin production and titration consisted of 2% tryptone, 1% glucose, 0.5% sodium chloride and 1.5% agar (7). For harvesting of endotoxin, C_9 cells were cultivated in a broth medium of the following composition per liter: sodium glutamate, 20 g; glucose, 5 g; Na_2HPO_4, 5.6 g; KH_2PO_4, 0.25 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; Ca $(NO_3)_2$, 10 mg; $FeSO_4 \cdot 7H_2O$, 50 μg. The sodium glutamate was filtersterilized and added to the remaining components which had been autoclaved at 15 psi for 30 min. The final pH of the medium was 7.6. The cells were grown in 15 liter fermenter jars containing 10 liters of medium for 18 hr at 37° in a New Brunswick fermenter, model FS-314. The culture was aerated at a rate of 2 liters of air per min and agitated by a paddle stirring at 300 rpm. The cells were then harvested by centrifugation and washed three times in distilled water.

Endotoxin extraction procedures. Endotoxin was extracted by two standard procedures. They were the aqueous phenol (8), and trichloroacetic acid (TCA) (9) methods. Both preparations were used in this study since they were found to be the most toxic as compared to preparations obtained by EDTA-lysozyme, (3), ethyl ether, (10), and hot water, (11), as previously described (12). The endotoxin obtained by these procedures was dialyzed against distilled water, purified by alcohol

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precipitation twice, washed in the cold with ethanol after each precipitation, and then lyophilized. The protein fraction from aqueous phenol or TCA preparations was obtained by the method of Goebel and Barry (13). Protein was also isolated from endotoxin obtained by the procedure of Homma and Suzuki (3).

Assay for pyocin activity of endotoxin. The two endotoxin preparations were tested for pyocin-like activity using suspensions of 500 μg and 1 mg dry weight/ml of sterile distilled water or 0.02 *M* tris (hydroxymethyl) aminomethane (Tris–HCl) buffer, (pH 7.5) containing 0.02 *M* magnesium chloride. The activity of the endotoxin preparations was assayed against 70 test strains by the method of Higerd *et al.* (7). Briefly, this consisted of the following procedure. The organisms were grown for 18 hr at 37° in tryptone–glucose–sodium chloride broth. The cells were harvested by centrifugation and resuspended in 0.85% saline to give an absorbancy of 0.10 at 660 nm in a Coleman Junior spectrophotometer. This stock suspension was then diluted 1:5 and 0.25 ml was added to the agar plates having the same composition as the broth. Seventy test strains were uniformly spread on the tryptone–glucose–sodium chloride agar medium with a glass rod spreader. The test strains consisted of 31 AP^+ (autoplaque positive) and 39 AP^- strains. After 3 hr incubation at 37°, 0.025 ml of each endotoxin preparation was spotted on the surface of the inoculated plates. Negative water and buffer controls were also spotted on the plates. The extracted protein from endotoxin was assayed by the same procedure and employed 500 μg/ml in water, and the Tris–HCl buffer.

Electron microscopy. Pyocin and endotoxin was prepared for electron microscopic observation and routine negative staining using the pseudoreplica technic of Baechler and Brandon (14) and Taylor & McCormick (15). The specimens were stained at room temperature for 15 sec employing 0.05% cobalt tungstate in distilled water, pH 6.8, (16) and observed with the RCA EMU-3E.

Results and Discussion. Since Homma and Suzuki (3) had previously reported that the protein component derived from *P. aeruginosa* endotoxin exhibited bacteriocinogenic activity (pyocin), our initial studies attempted to duplicate these earlier findings. However, no pyocinogenic activity was obtained with the protein when tested against 70 test strains. Endotoxin from the aqueous phenol and TCA preparations of C_9 were routinely used as a source for the protein component (8, 9). In addition, protein isolated from endotoxin obtained by the lysozyme-EDTA method of Homma and Suzuki (3) was used, but without success. The reason for these negative findings is unclear, but may possibly be due to differences in the strain of *P. aeruginosa* used. However, it should be pointed out that Eagon was also unable to find pyocin activity with the protein fraction (personal communication). Consequently, endotoxin obtained by both extraction procedures was tested for bacteriocinogenic activity against the 70 test strains using a concentration of 500 μg dry weight per ml. It was found that endotoxin obtained by the aqueous phenol method inhibited 6 out of 31 AP^+ strains. However, no inhibition was observed with the 39 remaining test strains which were all AP^-. Use of 1000 μg dry weight of endotoxin per ml gave the same pattern of results, but the zone of inhibition was wider. Similar results were obtained with the endotoxin obtained by the TCA method; however, 7 out of 31 AP^+ strains were inhibited. Inhibition was obtained with endotoxin suspended in both water and Tris-HCl buffer. Those strains which were inhibited by endotoxin exhibited zones of inhibition which varied in size from strain to strain and were also dependent upon the type of endotoxin preparation employed. In addition, good correlation between the 2 endotoxin preparations were obtained with 5 of the test strains which were sensitive to both preparations. As can be seen from Table I, strain 17 was inhibited only by the aqueous phenol endotoxin, while strain 41 was inhibited by only the TCA extracted endotoxin. At present, the reason for these two divergent reactions is not clear. In addition, it can also be seen from

TABLE I. Effect of Isolated Endotoxin from *P. aeruginosa* C_9 on Auto-Plaque Stimulation and Growth Inhibition.

		Ap-stimulation		Growth inhibition by endotoxin		Growth inhibition by protein from endotoxin	
Organism (AP^+)	Pyocin inhibition	aq. phenol:	TCA	aq. phenol:	TCA	aq. phenol:	TCA
Pa5	−	+	+	+	+	−	−
Pa13	+	−	−	−	−	−	−
Pa17	+	+	−	+	−	−	−
Pa29	+	−	−	−	−	−	−
Pa34	−	+	+	+	+	−	−
Pa40	+	−	−	−	−	−	−
Pa41	−	−	+	−	+	−	−
Pa43	−	+	+	+	+	−	−
Pa49	−	+	+	+	+	−	−
Pa50	−	−	+	−	+	−	−
L16	−	+	+	+	+	−	−
L19	+	−	−	−	−	−	−

Table I that only strain 17 was sensitive to the particulate pyocin particles as well as to one of the endotoxin preparations. Consequently, the antibacterial spectrum of endotoxin appears to be generally unrelated to that of pyocin particles. In addition to inhibition of bacterial growth, it was noted that the endotoxin preparations simultaneously enhanced auto-plaque formation in only those strains susceptible to inhibition. Enhancement of auto-plaque formation was signified by an increase in the number of individual auto-plaques as well as a wider zone of confluent lysis as compared with untreated AP^+ cultures. The appearance of increased auto-plaque formation was identical to that seen with antibiotics as previously described by Berk (6). Maximum lysis occurred in the periphery of growth closest to the clear zone of inhibition. No auto-plaque enhancement or growth inhibition was seen in AP^- strains, nor did the isolated protein fraction exhibit auto-plaque enhancement. Consequently, it would appear that the antibacterial activity of endotoxin was somehow related to the auto-plaque phenomenon. Also, the ability of endotoxin to act as a bacteriocin is consistent with the report that staphylococcin from *Staphylococcus aureus* is a bacteriocin composed of a lipoprotein-carbohydrate complex. Electron microscopic studies on the structure of staphylococcin (17) strongly resembles wall associated lipopolysaccharides from *P. aeruginosa* (16). The presence of bacteriocinogenic activity of endotoxin is also consistent with the previous report of Hamon (5) who was able to obtain both soluble and particulate bacteriocins from *P. aeruginosa.*

In an attempt to ascertain any further relationship of endotoxin with bacteriocinogenic properties of *P. aeruginosa,* purified, uncontracted pyocin particles from C_9 were suspended in 0.02 *M* tris(hydroxymethyl) aminomethane buffer (pH 7.5) containing 0.02 *M* magnesium chloride and incubated with endotoxin from C_9 and W_9 at 37° for 30 min. Electron microscopic examination of the mixtures indicated the following results: pyocin from C_9 did not adsorb to either endotoxin preparations from C_9 and remained in the "relaxed" or uncontracted state. However, pyocin particles adsorbed to both endotoxin preparations from the W_9 indicator strain underwent contraction as previously described (4). These results are consistent with that of Higerd *et al.* (4) who noted the identical response when intact cells or cell wall fragments from C_9 and W_9 were used for pyocin sensitivity studies. These results suggest that the re-

ceptor material for pyocin particles is endotoxin or chemically related material. This would be consistent with a previous study where the pyocin receptor substance was chemically analyzed and found to be composed mainly of lipopolysaccharide (18). Consequently, the current studies described herein indicate that endotoxin from *P. aeruginosa* behaves both as a receptor site for pyocin particles as well as a bacteriocinogenic factor. However, at the present time, the receptor site for the endotoxin preparations exhibiting pyocin activity is unknown and requires further elucidation.

Summary. Endotoxin from *Pseudomonas aeruginosa* exhibited bacteriocinogenic activity against several auto-plaque positive strains and appeared to enhance auto-plaque production in the inhibited strains, whereas all auto-plaque negative strains were unaffected. However, chemically removed protein moiety of the endotoxin did not exhibit any bacteriocinogenic activity when tested against seventy strains of *P. aeruginosa*. In addition, endotoxin appeared to behave as specific receptor site for bacteriocin particles (pyocin) obtained from *P. aeruginosa*. Consequently, when uncontracted pyocin particles from strain C_9 were incubated with endotoxin from the sensitive indicator strain W_9, they adsorbed to the endotoxin and underwent contraction. However, pyocin from C_9 did not adsorb or undergo contraction when incubated with endotoxin from C_9.

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