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Bacteriocin-like Material Produced by *Pasteurella pestis*

BY RENANA BEN-GURION AND I. HERTMAN

*Israel Institute for Biological Research,
Ness-Ziona, Israel*

SUMMARY: Strains of *Pasteurella pestis* under certain conditions form bacteriocin-like material, for which the name 'pesticin' is proposed; it inhibits the growth of *P. pseudotuberculosis*. The conditions necessary for induction of pesticin formation are similar to those which lead to induction of formation of some known bacteriocins. Of 24 strains of *P. pestis* tested, all but one produced pesticin. A mutant of *P. pseudotuberculosis* which was resistant to pesticin produced by one strain of *P. pestis* was found to be resistant to pesticin produced by all the other strains.

Reciprocal antibiotic actions were first described by Gratia (1925) and Fredericq (1946), who found that certain strains of *Escherichia coli* produce antibiotic substances that act specifically on other strains of the same species. These substances were named colicins (Fredericq, 1946). Further work showed that similar substances were also produced by many strains of *Shigella* (Fredericq, 1948*a*; Fastier, 1949), some strains of *E. freundii* (Fredericq, 1947), and a few *Salmonella* strains (Fredericq, 1952; Hamon, 1955). It was shown in 1952 (Jacob, Siminovitch & Wollman, 1952), that it was possible to induce formation of colicin by irradiation of the bacteria under conditions similar to those which cause induction in lysogenic bacteria. The colicins differ in the extent and specificity of their activity and in their physical and chemical properties (Fredericq, 1948*b*; Goebel, Barry & Shedlovsky, 1956; Hamon, 1956; Ludford & Lederer, 1953). Analogous substances were found in two other families of bacteria: pyocin, produced by *Pseudomonas aeruginosa* (Jacob, 1954), and megacin, produced by *Bacillus megaterium* (Ivanovics & Alföldi, 1955, 1957). The present paper describes the formation of bacteriocin-like material by strains of *Pasteurella pestis*; the substance will be referred to as 'pesticin'.

METHODS

Organisms. The following strains were used: A strain of *Pasteurella pseudotuberculosis* No. 134 C (kindly supplied by Professor H. B. Maitland, Department of Bacteriology, Manchester) and a streptomycin-resistant mutant of this strain. Strains of *P. pestis*: 8775, 8776, 8777, 8779, Bombay, TJW (NCTC), TRU Schuetz, Ts otten, P4, Pf2, TSR, P5, P3, P2, P12, L37 (received from the Department of Bacteriology, Hebrew University, Jerusalem), EV76, 248, H2, Elis, 195, 1122 (kindly supplied by Professor G. Girard, Institut Pasteur, Paris). Kimberley strain P (received from Instituto Oswaldo Cruz, Rio de Janeiro), strain P in a rough stage 'PR', a streptomycin-resistant mutant of

P. pestis strain TRU. A strain of *Shigella dysenteriae* (Shiga). A strain of *Escherichia coli* K12 (λ). A strain of *Pasteurella tularensis* strain Schu. Strains of *Salmonella typhimurium*, 156, 238, 243, 154, 246, 244.

Media. Tryptose broth (TS) consisted of Bacto Tryptose (Difco) 2 % (w/v), 0.5 % NaCl (w/v). Proteose peptone broth (Pr_3) consisted of Proteose peptone 3 (Difco) 2 % (w/v), NaCl 0.5 % (w/v), glucose 0.05 % (w/v), Na_2HPO_4 , 0.5 % (w/v). Blood agar base (BAB) consisted of: blood agar base (Difco) 4 % (w/v), defibrinated rabbit blood 3 % (w/v). Tryptose agar (TSA) and proteose peptone agar (Pr_3A) were prepared as described for liquid media with the addition of agar (Difco) 2 % (w/v). Phosphate buffer contained NaH_2PO_4 5 % (w/v), Na_2HPO_4 5 % (w/v) (pH=7). Trypsin solution: trypsin 1/300 (Nutritional Biochemical Corporation, Cleveland, Ohio) was dissolved in a buffer salt solution of the following composition (% w/v): NaCl, 0.8; KCl, 0.02; Na_2HPO_4 , 0.115; KH_2PO_4 , 0.02; $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.01; CaCl_2 , 0.01.

Conditions of cultivation. Stock cultures were maintained on BAB slopes. Broth cultures were started from 24 hr. blood agar slopes and incubated at 28°–30° for 20–24 hr. on a shaker.

Ultra-violet irradiation. The irradiation of 4 ml. samples in 9 cm. Petri dishes (less than 2 mm. layer of liquid) was carried out under constant agitation. A G15T8 hot Sterilamp (Westinghouse Electric Corporation, Bloomfield, N.J., U.S.A.) was used for irradiation. The intensity (measured with a SM-600 u.v. indicating meter for sterilamps) was 300 ergs $\text{sec}^{-1} \text{ cm}^{-2}$ at a distance of 87 cm. The same dose was used for irradiation of bacteria on solid media. After irradiation all further work was performed in dim light to avoid photo-reactivation.

Exposure to sonic vibration. Five to 10 ml. samples were exposed to sonic vibrations at 10 kc. in a 250 W. 'Raytheon' magnetostriction oscillator, Model DF 101 (Raytheon Manufacturing Co., Waltham, Mass., U.S.A.).

Optical density. The optical density of suspensions of organisms was measured with a Coleman Junior spectrophotometer at a wave length of 590 m μ .

Viable count. Samples (0.1 ml.) of an appropriate dilution of a culture were plated on BAB medium. The colonies were counted after incubation for 48 hr. at 30°.

Demonstration of pesticinogenic properties (two-layer method, Fredericq, 1954a). The bacteria tested for pesticin production were seeded in 1 ml. of Pr_3A on a basal medium (Pr_3A) layer. Immediately after the 1 ml. of agar solidified, a second layer of 10 ml. of Pr_3A was poured over the first and the plate incubated for 3 days at 30°. At the end of this period, when isolated colonies of the seeded culture appeared between the two layers of agar, the sterile surface of the upper layer was seeded evenly with the indicator strain, *Pasteurella pseudotuberculosis*. This was done by means of filter-paper which was soaked in a suspension of that strain. After 24 hr. further incubation at 37°, the indicator strain developed uniformly, except for some circular inhibition zones situated above the active colonies.

Pesticin titration. The pesticin was titrated by spotting drops (0.01 ml.) of a series of dilutions of the preparation to be titrated, on the surface of a plate

seeded with the indicator strain. The indicator plates were prepared some minutes before the titration by soaking a filter-paper in a suspension of the strain and leaving the paper on the plates for 5 min. The plates were then incubated at 37° for 24 hr. In this way a series of zones of decreasing inhibition was obtained ranging from complete inhibition through partial inhibition to normal growth. The highest dilution that gave a visible inhibition zone was defined as containing one arbitrary unit/ml.

RESULTS

Pesticin formation

The supernatant fluid from a well-aerated 48 hr. broth culture of *Pasteurella pestis*, grown at 30°, contained a substance (pesticin) which inhibited the growth of *P. pseudotuberculosis*.

After centrifugation of culture in the cold at 3000 rev./min. for 20 min. its supernatant fluid was treated as follows: (a) by addition of a few drops of chloroform to the supernatant, vigorous shaking for 10 sec., decantation followed by aeration of the liquid for 20 min. at 37° (to remove the residual chloroform); (b) by addition of 500 µg. dihydrostreptomycin (sulphate)/ml. and using as indicator a streptomycin-resistant mutant. Checks for sterility were performed after each treatment. These treatments did not destroy pesticin activity. Filtration of the supernatant fluid through ultra-fine sintered glass filters sometimes decreased the inhibitory effect.

When material from the agar surface of an inhibitory zone was transferred (bacteriological loop) to another plate with an indicator strain, no inhibition was seen. The inhibiting agent could not be transferred in series.

Transmission of pesticinogenic properties. By means of the two-layer method it was found that each bacterium in a population of a pesticin-producing strain was capable of transmitting this property to its descendants (Pl. 1).

Influence of media on activity. Experiments showed that the media on which pesticin was titrated considerably influenced the results. While one batch of TSA gave very good results, a different batch did not show any inhibition of *Pasteurella pseudotuberculosis* growth. No differences were found between different batches of Pr₃A. The media on which pesticin was titrated were always adjusted to pH 7.

In liquid media inhibition of *Pasteurella pseudotuberculosis* growth by pesticin was observed but no quantitative results could be obtained. All pesticin titrations described in this paper were performed on solid media (see 'Methods').

Temperature influence on pesticin activity. The activity of pesticin was 20 times higher when tested at 37° than at 30°. Table 1 shows the respective activities obtained in the titration of pesticin on plates incubated for 24 hr. at 37° and 30°.

It should be noted that *Pasteurella pseudotuberculosis* grows more abundantly at 37° than at 30° (unlike *P. pestis*).

As the pesticin was added directly on the indicator bacteria (*Pasteurella pseudotuberculosis*) and the presence and not the diameter of the inhibition zone was considered, no allowance was made for the variation in diffusion rate in agar resulting from differences in temperature.

Table 1. *Temperature influence on pesticin activity (activity expressed in arbitrary units)*

Samples of pesticin	Activity measured at 37°	Activity measured at 30°
1	400	20
2	1,000	50
3	2,000	75
4	10,000	200
5	8,000	300

Production of pesticin by different strains and spectrum of activity. Out of 24 strains of *Pasteurella pestis* tested, only strain TRU failed to produce pesticin. This strain was not sensitive to the pesticin produced by other strains of *P. pestis*. Attempts to transduce the pesticinogenic properties of other strains of *P. pestis* to strain TRU, by growing the two strains together, were not successful (cf. Fredericq, 1954b). *P. tularensis* strain Schu., *Escherichia coli* K12 (λ), *Shigella dysenteriae* (Shiga), and 6 strains of *Salmonella typhimurium*, were not sensitive to pesticin. A *P. pseudotuberculosis* mutant, resistant to the pesticin produced by one strain of *Pasteurella*, was resistant to the pesticin produced by all other strains.

Chemical and physical properties of pesticin

Nature of pesticin. Pesticin is sensitive to proteolytic enzymes. Its activity is completely destroyed by incubation with 0.025 % (w/v) trypsin for 20 min. (1/300 NBC) at 37° (pH 7). It has a relatively high molecular weight since it diffuses slowly in agar and does not pass through cellophan.

Table 2. *Effect of heat on pesticin activity (pesticin activity expressed in arbitrary units)*

Original value 2000		
Time of heating	In boiling water	At 60°
1 min.	100	—
3 min.	50	—
5 min.	50	—
15 min.	2	—
1 hr.	—	50
2 hr.	—	25
3 hr.	—	2

Heat. Pesticin is thermolabile, its activity is decreased by more than 95 % through heating for 5 min. in boiling water. A similar effect is obtained by heating to 60° for 3 hr. Table 2 gives an example of the effect of heat on pesticin. Most of its activity is lost after one day at room temperature (30°)

and it cannot be kept for more than a few days in an ordinary refrigerator. Storage for 3 months at -20° did not decrease its activity.

pH. Pesticin is stable between pH 6 and 8; outside this range its activity diminishes. Table 3 shows the effect of variation in the pH of the media (Pr_3), containing the pesticin, on its activity. The media containing pesticin were kept for 2 hr. at 37° at various pH values. Part of the medium was brought back to its original pH (7), while the remainder was kept at the altered pH value. Titration was then carried out in the usual manner on both parts. Where the titration of pesticin was carried out at a pH different from 7, the dilution solution (Pr_3) was adjusted to the identical pH value. Where the pH which was outside 6–8 range was brought back to 7, the activity did not return to its previous value.

Table 3. *Activities of pesticin at different pH values*
(activity measured in arbitrary units of pesticin)

pH	Titration without further alteration in pH	Titration after readjustment to pH 7
12	2	2
11	100	100
10	800	800
9	1000	1000
7	2000	2000
5	1000	1000
4	400	400
3	100	100
1.5	50	50

U.v. irradiation. Pesticin is very resistant to u.v. irradiation. No decrease in activity was detected after irradiation for 75 min. at $300 \text{ erg. sec.}^{-1} \text{ cm.}^{-2}$.

Adsorption. Pesticin is completely adsorbed by Seitz filters and partly by sintered glass filters.

Induction of pesticin biosynthesis

The formation of pesticin by strains of *Pasteurella pestis* could be induced by u.v. irradiation. The conditions of pesticin induction were found to be similar to the conditions for induction of other bacteriocins in bacteriocinogenic bacteria (Kellenberger & Kellenberger, 1956; Hamon & Lewé, 1955).

Liquid cultures (24 hr.) of *Pasteurella pestis* in media TS or Pr_3 , of optical density ≥ 90 , did not contain pesticin. When these cultures were exposed to sonic vibrations for 20 min., 88 % of the bacteria were disintegrated, but no pesticin was found in the culture fluid. A similar medium containing pesticin, subjected to the same degree of sonic vibration for 20 min., did not show any decrease in the activity of pesticin.

When 24 hr. cultures of *Pasteurella pestis* were irradiated with a suitable dose of u.v. radiation pesticin appeared in the medium. An example of pesticin formation after u.v. irradiation is shown in Fig. 1. In this and similar

experiments it was observed that after irradiation, pesticin appeared in the medium after 15 min. at 37°, and only after 30 min. at 30°. (The optimum temperature for growth of *P. pestis* is 30°.)

The amount of pesticin liberated into the medium increased with time; at 37° it reached the maximum 2 hr., and at 30° in 3 hr. after irradiation. The irradiation itself caused a decrease in viable bacterial count of 50 % (irradiation for 90 sec.). No decline in bacterial count during incubation of the irradiated culture was detected.

Fig. 1. Pesticin formation after u.v. irradiation of a culture of *Pasteurella pestis*, strain Kimberley (P), grown and irradiated in Pr_3 medium. The times of irradiation were zero and 90 sec. optical density 98. Pesticin concentration expressed in arbitrary units per ml. A, Count of non-irradiated bacteria at 37°; B, count of non-irradiated bacteria at 30°; C, count of irradiated bacteria at 37°; D, count of irradiated bacteria at 30°; E, pesticin formation at 37°; F, pesticin formation at 30°; G, pesticin formation at 0°.

Irradiation of organisms on solid media

Microcolonies of *Pasteurella pestis* on medium Pr_3A formed after incubation for 20 hr. were irradiated for 90 sec. and then tested for pesticin formation by the double-layer method. Inhibition zones appeared without visible bacterial growth of *P. pestis* (Pl. 1, fig. 2). No inhibition zones were formed when the

bacteria on agar were killed by exposure to chloroform vapour immediately after irradiation. Exposure of the bacteria to chloroform vapour 2 hr. after irradiation did not affect the formation of the inhibition zones.

Pesticin formation in buffer

Pasteurella pestis, grown for 24 hr. in medium Pr₃, was washed twice with phosphate buffer and then irradiated (90 sec.). Immediately after irradiation one part of the bacterial suspension was diluted 1/4 in Pr₃ medium, and another part was similarly diluted in phosphate buffer and both suspensions were incubated at 37°. After 2 hr. of incubation, pesticin was formed only in the Pr₃ broth; there was no formation of pesticin in phosphate buffer.

Inducibility of different strains of Pasteurella pestis. U.v. irradiation induced the production of pesticin by all the strains which could produce it spontaneously. Strain TRU which did not form pesticin spontaneously, did not produce it after u.v. irradiation.

DISCUSSION

The production of antibiotic material which inhibits the growth of *Pasteurella pseudotuberculosis* seems to be a common phenomenon in the species *P. pestis*; 23 out of 24 strains tested (or 22 out of 23 strains, if strain EV and ELIS are identical; Girard, 1957) produced pesticin.

The properties of pesticin allow it to be distinguished from classical antibiotics and to be classified with other bacteriocins, i.e. protein-like substances produced by bacteria which inhibit specifically related strains. The formation of some bacteriocins can be induced by ultraviolet irradiation. While generally as a result of induced formation of other bacteriocins bacteria are lysed (Jacob *et al.* 1952; Ivanovics & Alföldi, 1957; Jacob, 1954), pesticin formation after u.v. irradiation is not accompanied by total lysis. It is possible that only a very small proportion of the population produces pesticin and undergoes lysis. The methods used here (viable count and spectrophotometry) are not sensitive enough to detect a change in less than 10 % of the population. It is therefore possible that production of pesticin is accompanied by lysis of a small proportion of the population. According to some authors (Fredericq, 1954c; Hamon & Lewé, 1955; Kellenberger & Kellenberger, 1956), however, induction of colicin formation does not result in lysis, unless the strains are also lysogenic. Kellenberger & Kellenberger (1956) examined 63 colicinogenic strains of *Escherichia coli* and concluded that strains that were only colicinogenic did not undergo lysis after u.v. irradiation, while a complete or partial lysis was always due to the presence of developing bacteriophage in the bacteria. After irradiation of colicinogenic, but not lysogenic bacteria, a residual growth of the bacteria was observed, and this was followed by an apparently stationary phase in the growth, while the production of colicin continued (Hamon & Lewé, 1955). In the case of pesticin formation following irradiation, no stationary phase in the growth curve was observed. The bacterial count increased during pesticin formation. It is quite possible that the expected plateau was masked by the growth of non-induced bacteria.

When bacteria on agar plates are induced by u.v. irradiation to form pesticin, they do not form colonies. This may indicate that pesticin synthesis is a lethal biosynthesis, and that the growth of organisms in irradiated liquid medium might be due to the growth of organisms which were not affected by irradiation.

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Fig. 1

Fig. 2

R. BEN-GURION & I. HERTMAN—PESTICIN. PLATE 1

(Facing p. 297)

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EXPLANATION OF PLATE

- Fig. 1. Inhibition zones of *Pasteurella pseudotuberculosis* above colonies of *P. pestis*.
- Fig. 2. Inhibition zones of *Pasteurella pseudotuberculosis* produced by irradiation of micro-colonies of *P. pestis*.

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