PESTICINS

II. Production of Pesticin I and II

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ABSTRACT

BRUBAKER, ROBERT R. (Fort Detrick, Frederick, Md.) AND MICHAEL J. SURGALLA. Pesticins. II. Production of pesticin I and II. J. Bacteriol. **84**:539–545. 1962.—Pesticin I was separated from pesticin I inhibitor by ion-exchange chromatography of cell-free culture supernatant fluids and by acid precipitation of soluble preparations obtained from mechanically disrupted cells. The latter procedure resulted in formation of an insoluble pesticin I complex which, upon removal by centrifugation and subsequent dissolution in neutral buffer, exhibited a 100- to 1,000-fold increase in antibacterial activity over that originally observed. However, activity returned to the former level upon addition of the acid-soluble fraction, which contained pesticin I inhibitor. Since the presence of pesticin I inhibitor leads to serious errors in the determination of pesticin I, an assay medium containing ethylenediaminetetraacetic acid in excess Ca^{++} was developed; this medium eliminated the effect of the inhibitor. By use of the above medium, sufficient pesticin I was found to be contained within 500 nonirradiated cells to inhibit growth of a suitable indicator strain; at least 10^7 cells were required to effect a corresponding inhibition by pesticin II. Although both pesticins are located primarily within the cell during growth, pesticin I may arise extracellularly during storage of static cells. Slightly higher activity of pesticin I inhibitor was found in culture supernatant fluids than occurred in corresponding cell extracts of equal volume. The differences and similarities between pesticin I and some known bacteriocins are discussed.

Prophages, the F factor, and colicinogenic determinants, termed episomic elements by Jacob and Wollman (1958), are infective particles which, when present in a bacterial cell, may replicate independently within the cytoplasm or remain as integrated units attached to the chromosome. The current concept of colicinogeny has evolved from interpretation of experimental data concerning colicin E (*see* Jacob and Wollman, 1961). The colicin determinant controls the production of an antibacterial protein or colicin; these substances are active against strains of bacteria within or closely related to the species from which the colicin is derived. A strain sensitive to a colicin usually lacks the corresponding colicin determinant but always possesses the corresponding receptor site (Fredericq, 1958). Most colicins are not considered to be normal constituents of cells which possess the genetic potential to synthesize these substances; however, a colicin may be spontaneously produced by a minority of the cells in a culture (Fredericq, 1958), or may be induced by exposure to ultraviolet light (Jacob, Siminovitch, and Wollman, 1952). The term colicin is reserved for antibacterial substances of the type described above that are produced by certain strains of *Escherichia*, *Salmonella*, and *Shigella*. The general term bacteriocin (Jacob et al., 1953) encompasses colicins as well as analogous or similar substances such as pyocin (Jacob, 1954), megacin (Ivanovics and Alfoldi, 1954), staphylococcins (Fredericq, 1946), and pesticin (Ben-Gurion and Hertman, 1958), which are produced by *Pseudomonas aeruginosa*, *Bacillus megaterium*, staphylococci, and *Pasteurella pestis*, respectively. Other species of the order *Eubacteriales*, as well as of the order *Myxobacteriales* (Anaker and Ordal, 1959), have been reported to produce bacteriocins.

The discovery by Ben-Gurion and Hertman was of interest because there are some indications that an episomic element in *P. pestis* may be associated with the genetic control of virulence (Surgalla, 1960). The bacteriocin-like substance or pesticin described by these workers is active against the more common type I strains of *Pasteurella pseudotuberculosis* (Burrows and

Bacon, 1960) and against certain strains of *E. coli* (Brubaker and Surgalla, 1961; Smith and Burrows, 1962). This activity was termed pesticin I upon the discovery of a second bacteriocin-like substance, designated pesticin II, which is active against a few strains of *P. pestis* that fail to produce the pesticin of Ben-Gurion and Hertman; pesticin II is produced by all tested strains of *P. pestis* and *P. pseudotuberculosis*. In addition, a substance was found that inhibits the activity of pesticin I in the presence of iron; this compound, termed pesticin I inhibitor, is produced by strains of both *P. pestis* and *P. pseudotuberculosis* (Brubaker and Surgalla, 1961).

The purposes of this paper are to describe a discrepancy between the real and apparent production of pesticin I and to discuss some similarities and differences between this substance and some known bacteriocins.

MATERIALS AND METHODS

The avirulent pesticinogenic strain A1122 and its nonpesticinogenic mutant strain A12 were employed as a source of pesticin I (PI) and pesticin I inhibitor (PIi), respectively. *P. pseudotuberculosis* strain PB1/− was used as an indicator for PI, and *P. pestis* strain Java was employed as the indicator for pesticin II (PII). These strains have been described previously (Brubaker and Surgalla, 1961). Pesticin titers were determined on Difco Blood Agar Base (BAB), which contained various added reagents, by the following modification of the method employed by Ben-Gurion and Hertman (1958). Petri dishes containing 25 ml of hardened agar medium were spotted with drops from serially diluted test samples. After the agar surface dried, the plates were treated for 5 min with chloroform vapor, overlayered with 5 ml of seeded agar, and incubated for 48 hr at 37 C. Activity was recorded in terms of the unit defined by Ben-Gurion and Hertman.

Cultures were prepared by washing the growth from a BAB slant into 100 ml of Difco Heart Infusion Broth (HIB) plus 1.0% xylose contained in a 2-liter flask, which was aerated on a shaker at either 26 or 37 C for 36 to 48 hr. Cells were removed by centrifugation at 27,000 × *g* for 30 min, washed in 0.033 M potassium phosphate buffer (pH 7.2), and finally suspended at a concentration of 10^{10} to 10^{11} cells per ml in distilled water or phosphate buffer. These suspensions were acetone-dried, disrupted in a 10-kc Raytheon sonic oscillator, or treated at 20,000 psi in a French pressure cell. Since no significant amount of free PI was associated with the insoluble residue, cellular debris was usually removed from the disrupted preparations by centrifugation. However, when intra- and extracellular activities were compared, the cells were quantitatively removed from the culture by centrifugation prior to disruption. The resulting extract, containing cellular debris, was diluted to the volume of the original culture. Activities of the diluted cell preparations and their culture supernatant fluids were then compared.

Both manganese ethylenediaminetetraacetic acid (Mn-EDTA) and calcium ethylenediaminetetraacetic acid (Ca-EDTA) were prepared from the tetrasodium salt (Eastman Kodak Chemical Co.) at a calculated 5% excess of cation. The buffer employed in chromatographic separations on diethylaminoethanol (DEAE) cellulose (Eastman Kodak Chemical Co.) was 0.01 M tris(hydroxymethyl)aminomethane-HCl, pH 7.6 (tris). Reagent-grade chemicals were employed in all experiments.

RESULTS

Effect of PIi. Ben-Gurion and Hertman (1958) were unable to detect PI in liquid cultures after exposure to sonic oscillation for 20 min. Contrary to these results, we were often able to detect this substance after sonic oscillation of unwashed cells. In addition, activity of PI was always detected in sonic-treated preparations or pressure-cell extracts of previously washed cells, and in powders of acetone-dried bacilli. Employing BAB as the assay medium, we found that extracellular activity was often present in young cultures grown at 26 C, but was rarely observed in cultures grown at 37 C, regardless of the period of incubation. Intracellular activity was always high and was independent of the age of the culture or its temperature of incubation. One explanation for the results of these preliminary experiments might be that PIi, a substance known to suppress the activity of PI (Brubaker and Surgalla, 1961), was present in those samples which exhibited no antibacterial activity. If suppression of PI activity by PIi occurs in liquid media, it would become necessary to develop an assay method which would eliminate this effect before quantitative deter-

mination of PI could be performed. A definite inhibitory effect by PIi was indicated by the following experiments.

Cell-free supernatant fluids of 48-hr cultures of strain A1122, which contained no detectable PI, exhibited this activity upon addition of DEAE cellulose. The following experiment was performed to clarify this phenomenon. A 50-ml sample of a culture supernatant of strain A1122 was dialyzed for 6 hr against cold tris buffer. The dialyzed sample was then added to 2.5 g of DEAE cellulose contained in a column 2.0 cm in diameter. The column was washed with 200 ml of tris buffer, and a 50-ml sample of the eluate containing the nonadsorbed matter was collected and labeled fraction A. Tris buffer containing 1.0 M NaCl was then added to the column, and 50 ml of this eluate were collected and labeled fraction B. The above procedure was repeated with a culture supernatant of the nonpesticinogenic strain A12. Results of qualitative determinations of PI activity in the dialyzed culture supernatant fluids and in the A and B fractions are given in Table 1. PI was detected in fraction A but not in the starting sample nor in fraction B of strain A1122; as expected, all samples of strain A12 were negative. Upon adding a portion of each inactive sample to an equal volume of fraction A of strain A1122, no PI activity was observed except in the case of fraction B of strain A12. Thus, the activity of PI is reversibly suppressed by a substance which, under the above conditions, may be adsorbed on DEAE cellulose, whereas all of the PI is not. The explanation for the occurrence of this inhibitor in the fraction B of strain A1122 but in the fraction A of strain A12 may be more subtle than that of differential displacement.

Filter-paper strips were soaked with the strain A12 and strain A1122 culture supernatants and fraction A and B of the latter strain. The strip soaked in the active fraction A was placed on the surface of a petri plate containing 25 ml of BAB. This strip was then overlayered with 5 ml of seeded agar, and the remaining strips were cross-layered on the agar surface. Upon subsequent incubation at 37 C for 48 hr, colonies were observed in the immediate vicinity of the strips overlaying the strip containing PI. Since this reaction is identical to that previously described for PIi (Brubaker and Surgalla, 1961), the presence of this substance was assumed.

TABLE 1. *Expression of pesticin I activity in samples prepared by chromatography of culture supernatants on DEAE cellulose**

Sample tested (strain)	Activity of PI
Culture supernatant (A1122, A12)	0
Fraction A (A1122)	+
Fraction A (A12)	0
Fraction B (A1122, A12)	0
Fraction A (A1122) plus culture supernatant (A1122)	0
Fraction A (A1122) plus fraction B (A1122)	0
Fraction A (A1122) plus culture supernatant (A12)	0
Fraction A (A1122) plus fraction A (A12)	0
Fraction A (A1122) plus fraction B (A12)	+
Fraction A (A1122) plus saline control	+

* Fraction A = nonadsorbed material in 50 ml of culture supernatant; fraction B = adsorbed matter eluted by 1.0 M NaCl in tris buffer.

Subsequent tests indicated that a marked increase over the endogenous PI activity of pressure-cell extracts or sonic-treated cells occurs upon treatment with DEAE cellulose. However, this resin has little capacity to remove PIi from concentrated solutions.

Excellent separation of PI from PIi in preparations of mechanically disrupted cells was obtained by precipitation of PI by the addition of 1.0 N HCl to yield a pH of 4.7. The insoluble matter was removed by centrifugation and dissolved to its original volume in phosphate buffer. The acid-soluble fraction was neutralized with a few drops of 1.0 M NaOH. Equal volumes of the two samples were added together, and the remainders, as well as a part of the untreated sample, were diluted 1:2 in phosphate buffer. Upon subsequent test on BAB, titers of 10^5, 10^8, 10^2, and 10^5 units per ml were obtained for the starting, acid-insoluble, acid-soluble, and mixture of acid-insoluble and soluble samples, respectively. Thus, the separation of Pi from PIi resulted in a dramatic increase in activity, which was reduced to that originally observed upon return of the sample containing PIi. Subsequent experiments indicated that this increase was of the order of 100- to 1,000-fold.

As suggested previously (Brubaker and Surgalla, 1961), these results might be explained by the probability that PI is a basic protein

(isoelectric point ~7.5, estimated from paper-curtain electrophoresis data). Upon reduction of the pH of a soluble preparation of disrupted *P. pestis* cells, the PI may be precipitated as the insoluble nucleate, leaving the acid-soluble PIi in solution. After centrifugation, the return to neutral pH presumably results in dissociation of the salt complex, with liberation of free PI at an apparently increased titer.

We reasoned that an estimation of PIi might be obtained by adding this substance to a known quantity of PI and determining the resultant loss of antibacterial activity. Accordingly, an acid-soluble fraction of strain A1122, assumed to be free of PIi, was diluted to contain 2×10^6 units of PI per ml. Samples of this standard were tested by (i) dilution in a 48-hr culture supernatant or pressure-cell extract of strain A12, or (ii) by diluting in HIB after an equal volume of either of the above was added. The pressure-cell extracts were diluted to the volume of the culture supernatants before the assays were performed. The results of this experiment are given in Table 2. No antibacterial activity was observed when the standard PI sample was diluted in the strain A12 culture supernatant; however, 10^2 units of PI were still detectable when the strain A12 pressure-cell extract was employed as diluent. Similar results were obtained with the nonpesticinogenic strain Java grown at 26 and 37 C. Thus, it appears that nonpesticinogenic cells, and presumably pesticinogenic cells, elaborate more free PIi into the culture medium than is retained intracellularly.

TABLE 2. *Inhibition of pesticin I activity in the presence of pesticin I inhibitor*

Addition (1 volume) to standard PI sample	Diluent	Units PI per ml
HIB*	HIB	10^6
HIB	Strain A12 culture supernatant	<10
HIB	Strain A12 pressure-cell extract	10^2
Strain A12 culture supernatant	HIB	10^6†
Strain A12 pressure-cell extract	HIB	10^6†

* Heart Infusion Broth.

† Partial activity, as expressed by incomplete zones of inhibition.

TABLE 3. *Titers of intra- and extracellular pesticin I produced by strain A1122 cells grown in Heart Infusion Broth plus 1.0% xylose**

Addition to basal layer of Blood Agar Base†	26 C-grown culture		37 C-grown culture	
	Supernatant	PCE‡	Supernatant	PCE
None	10^1	10^4	10^0	10^4
Ca^{++}	10^2	5×10^4	10^1	10^5
Mn^{++}	10^1	5×10^4	10^1	10^5
Mn-EDTA	10^1	5×10^4	10^1	10^5
Ca^{++} plus Mn^{++}	10^2	5×10^4	10^1	10^5
Ca^{++} plus Mn-EDTA	10^3	5×10^6	10^2	10^7
Mn^{++} plus Mn-EDTA	10^2	10^4	10^1	10^4
Ca^{++} plus Mn-EDTA plus Mn^{++}	10^2	5×10^4	10^1	10^5

* Results are expressed as the reciprocal of the highest dilution which exhibited activity.

† Ca^{++}, 0.01 M; Mn^{++}, 0.005 M; Mn-EDTA, 0.01 M.

‡ Pressure-cell extract of washed cells diluted to the corresponding volume of the culture supernatant.

When an equal volume of the standard PI was admixed with the strain A12 preparations and subsequently diluted in HIB, the expected decrease in titer was not observed. Instead, only partial activity was obtained, as evidenced by incomplete zones of inhibition. Therefore, the suppression of PI activity by PIi is not stoichiometric. It is possible that a suitable method for the determination of PIi might be developed by dilution of this substance in a constant amount of PI; the definition of the kinetics of this reaction is a problem deserving of further study.

Determination of PI. The above experiments demonstrated the necessity of developing an assay method for PI that eliminates the undesirable effect of PIi. Certain substances, most notably Ca^{++}, Mn^{++}, and metal-chelating agents, are known to enhance the activity of PI by presumably distinct mechanisms; in addition, Fe^{+++} enhances the activity of PIi (Brubaker and Surgalla, 1961). A number of potential assay media were tested by spotting drops of serially diluted culture supernatants and pressure-cell

TABLE 4. *Suppression of pesticin I inhibitor activity in various intracellular fractions of strain A1122 upon assay on CCE medium*

Test sample	Pesticin activity*	
	BAB	CCE
Pressure-cell extract	3×10^5	5×10^8
Acid-soluble fraction	4×10^1	5×10^2
Acid-insoluble fraction†	5×10^7	6×10^7
Acid-soluble plus acid-insoluble fraction	2×10^5	5×10^7

* Units of pesticin I per ml of sample; BAB = Blood Agar Base; CCE = Blood Agar Base plus 0.01 M Ca^{++} plus 0.5% Ca-EDTA.

† Assumed to be free of PIi.

extracts of 26 and 37 C-grown cultures of strain A1122 onto BAB containing 0.01 M Ca^{++}, 0.005 M Mn^{++}, or 0.01 M Mn-EDTA, added either singly or in combination. After drying, all plates were overlayered with 5 ml of seeded BAB prior to incubation. Regardless of the assay medium employed, the titers of the culture supernatants were uniformly low compared with those of the pressure-cell extracts (Table 3). The addition of any single reagent had only a slight effect; however, Mn-EDTA plus Ca^{++} acted synergistically to yield titers approximately 2 logs higher than those which were observed when no addition was made. The incorporation of 0.005 M Fe^{+++} into any of the test media resulted in complete loss of PI activity, even in cases where sufficient Mn-EDTA was theoretically present to exchange with all of the added Fe^{+++}. Differences in titers obtained for cells grown at 26 and at 37 C are not considered to be significant. Since excess Mn^{++} did not enhance activity in the presence of Mn-EDTA and Ca^{++}, Mn^{++} could be eliminated from the medium, permitting the substitution of Ca-EDTA for the manganese chelate. Subsequent investigation demonstrated that use of an assay system composed of BAB plus 0.01 M Ca^{++} and 0.5% Ca-EDTA (CCE), employed in both basal and top-seeded agar layers, resulted in approximately 1 log higher activity than that observed when Mn-EDTA and Ca^{++} were used solely in the basal layer.

The following experiment was performed to demonstrate that the increased titer observed on CCE, as compared with that obtained on BAB, was due to suppression of the activity of PIi. An acid-soluble and an acid-insoluble fraction of a pressure-cell extract of strain A1122 were prepared as described above and tested on BAB and on CCE. Identical titers were obtained on both assay media in the case of the acid-insoluble sample, which is presumed to be free of significant PIi (Table 4). When the acid-soluble sample containing PIi was added to the acid-insoluble sample containing the majority of PI, the normal decrease in titer was observed on BAB but not on CCE, thus demonstrating that PIi is without effect on the latter medium. A comparison of titers on CCE of the starting sample and the acid-insoluble fraction indicates that about 1 log of the PI activity is lost upon treatment with acid. The reason why an analogous decrease was not observed on BAB is not known.

Production of pesticins. With the development of CCE, a medium which by virtue of its Ca^{++} content exhibits excellent activity of PII (Brubaker and Surgalla, 1961), we were in a position to determine quantitatively both pesticin activities at various phases of growth. A BAB slant was inoculated with strain A1122, and, after incubation for 24 hr at 26 C, the growth was suitably diluted and inoculated at 10^6 cells per ml into flasks which were incubated at 26 C as described previously. With each tenfold increase in bacterial growth, a sufficient volume of culture was harvested and centrifuged to yield at least 10^{10} packed cells. The cells were washed in phosphate buffer, resuspended in buffer, and treated to sonic disruption for 5 min; the treated suspension was then diluted with buffer to the volume of the original culture. The extra- and intracellular PI and PII concentrations were then determined on CCE by titration of the culture supernatants and their corresponding sonic-treated suspensions. A constant intracellular PI titer of 2×10^6 units per 10^9 cells (1 unit per 500 cells) was observed; approximately 10^7 cells were required before PII activity was obtained (Table 5).

During the course of these experiments, we observed that cells which had been harvested by centrifugation, washed, and stored for 72 hr at 5 C in phosphate buffer contained only 20% of the intracellular PI originally present. Subsequent testing demonstrated that approximately 75% of the missing PI could be accounted for in the buffer. Although viability was reduced upon

TABLE 5. *Intra- and extracellular pesticin I and II concentrations of strain A1122 cells tested on CCE medium* at various phases of growth at 26 C in Heart Infusion Broth plus 1.0% xylose*

Time of harvest	Viable count per ml	Units of pesticin per ml of culture				Units of intracellular pesticin per 10^9 cells	
		Culture supernatant		Sonic extract of washed cells			
		PI	PII	PI	PII	PI	PII
hr							
12	2.3×10^7	0	0	5×10^4	0	2.2×10^6	—
20	8.9×10^7	1	0	2×10^5	0	2.9×10^6	—
36	3.0×10^9	10	0	8×10^6	1	2.7×10^6	3
48	1.5×10^{10}	10	0	3×10^7	10	2.0×10^6	7

* CCE = Blood Agar Base plus 0.01 M Ca^{++} plus 0.5% Ca-EDTA.

storage, microscopy failed to detect the presence of sufficient cell lysis to account for this increase of extracellular activity.

DISCUSSION

Some observations which may have prompted Ben-Gurion and Hertman (1958) to consider PI as a bacteriocin-like substance are as follows. (i) No antibacterial activity was observed in a whole culture which had been subjected to sonic oscillation; thus, the presence of endogenous PI in nonirradiated cells was discounted. (ii) Of the potential indicator species tested, only the closely related *P. pseudotuberculosis* species was inhibited by PI. (iii) Since PI activity was observed in culture supernatants upon irradiation of cells subsequently transferred to a rich medium, the components of these media were presumed to be involved in *de novo* pesticin biosynthesis. (iv) Certain nonpesticinogenic mutants appeared to be sensitive to PI (Hertman and Ben-Gurion, 1958).

Since additional data have become available, the above points will be briefly reviewed in order. (i) It is possible that Ben-Gurion and Hertman failed to detect PI in sonic-treated whole cultures because of the presence of extracellular PIi, which may partially inhibit the activity of as much as 10^6 units of PI per ml (Table 2). We found that PI is always detected in sonic-treated preparations, provided that the cells are washed prior to disruption. (ii) Subsequent investigation has shown that cells of *E. coli* may also be killed by PI; this species is classified in a family distinct from that of the genus *Pasteurella*. However, as noted by many workers, *P. pestis* shares many properties in common with the enteric group of bacteria. (iii) An extracellular titer of 5×10^2 units of PI per 7.5×10^6 cells was obtained by Hertman and Ben-Gurion upon suspension of irradiated cells into a complex medium. Our data indicate that at least 30 times this amount of PI is present within nonirradiated cells, suggesting that the nutritional requirements for pesticin synthesis tabulated by these workers might equally well be associated with PI transport, analogous to that observed in presumably intact stored cells. (iv) These workers presented no evidence that their nonpesticinogenic mutants were sensitive to PI rather than to the activity subsequently described as PII. The latter may be the case, since the loss of PI production is associated with the acquisition of sensitivity to PII. In addition, strains have been obtained which produce PI and are also sensitive to this substance (Brubaker and Surgalla, 1961).

The antibacterial activity of PI and its sensitivity to trypsin are the only properties which indicate that this substance may be a bacteriocin. Since these two properties may also be possessed by other bacterial products, such as lytic enzymes, it becomes necessary to re-evaluate the status of PI. It is apparent that pesticinogeny is not analogous to the concept of colicinogeny, as described for colicin E. However, since other colicins also deviate from this model, it is impossible to state that PI is not a bacteriocin. For example, in one *E. coli* strain, colicin K is also an integral part of the bacterial cell; this substance is not induced by ultraviolet light but is normally released into culture media maintained at neutral pH (Rude and Goebel, 1961; Goebel, Barry, and Shedlovsky, 1956). Despite the

absence of suitable gene-transfer mechanisms in *P. pestis*, it is possible that either the demonstration of an additional biochemical activity of PI, or a study of its mode of action, may result in a more suitable classification. In view of the data presented above, plus the finding that strains of *P. pseudotuberculosis* may possess the same critical virulence properties as do strains of *P. pestis* (Burrows and Bacon, 1960), the hypothesis that the PI determinant may be associated with the control of virulence has been abandoned. However, the possibility that PI may play a role in the pathogenic process is being considered.

The role of PIi and the associated effect of iron remain obscure; PIi may play no role in a process analogous to cellular immunity, since this substance is produced by strains which are sensitive to PI. It is conceivable that further study of known bacteriocin systems will reveal analogous phenomena. The transitory resistance described by Mukai (1960) may possibly be caused by a substance with biological properties similar to those of PIi.

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