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A Study on Pesticin Biosynthesis

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SUMMARY: Strains of *Pasteurella pestis* under certain conditions form a bacteriocin-like material, which has been named pesticin. Pesticin formation was induced by ultraviolet irradiation. A chemically defined medium composed of inorganic salts and amino acids supported the synthesis of pesticin by irradiated organisms. The minimal amino acid requirement for pesticin synthesis has been established. For the synthesis of pesticin at 37° the irradiated organisms required certain amino acids which they did not require at 27°. Bacteria in the stationary phase and actively dividing bacteria were equally able to produce pesticin upon irradiation. Concentrations of chloramphenicol lower than those required for bacteriostasis inhibited pesticin synthesis. Non-pesticin producing mutants of *P. pestis* were obtained by exposure of the bacteria to higher doses of u.v. irradiation than those required for the induction of pesticin formation. These mutants were sensitive to pesticin.

It was reported previously (Ben-Gurion & Hertman, 1958) that ultraviolet (u.v.) irradiated *Pasteurella pestis* produces a bacteriocin-like material which we named pesticin, when incubated in Proteose peptone broth, while no pesticin was detected after incubation in phosphate buffer. In view of the apparent protein nature of pesticin (Ben-Gurion & Hertman, 1958) the way of its production can be considered as a problem in protein synthesis.

Pasteurella pestis exhibits different amino acid requirements for growth in defined media at 27° as compared with 37° (Hills & Spurr, 1952; Higuchi & Carlin, 1958). The synthesis of a number of antigens of *P. pestis* is also known to be temperature dependent (Crompton & Davies, 1956; Fox & Higuchi, 1958). The present study has been mainly concerned with the replacement of the complex proteose peptone medium, in which pesticin synthesis has been observed, by a chemically defined medium.

METHODS

Organisms. The following strains were used: a streptomycin-resistant mutant of *Pasteurella pestis* Kimberley strain P; and *P. pseudotuberculosis* No. 134 C.

Media. Proteose peptone broth (Pr3); Proteose peptone agar (Pr3A); blood agar base (BAB); phosphate buffer m/15. These media were prepared as described previously (Ben-Gurion & Hertman, 1958).

Casein hydrolysate mineral glucose medium (CHMG) (Englesberg & Levy, 1954) had the following composition (% w/v): vitamin-free Casamino acids (Difco), 3; glucose, 0.2; NH_4Cl , 0.1; CaCl_2 , 0.001; $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 0.00025; $\text{MgSO}_4 \cdot 3\text{H}_2\text{O}$, 0.035; $\text{KH}_2\text{PO}_4 + \text{Na}_2\text{HPO}_4$ buffer, pH 7, 0.025 M. The

Casamino acids were dissolved in distilled water. Glucose was sterilized separately by autoclaving and added aseptically.

Amino acid media. The composition of these media was otherwise that of the CHMG medium, but the vitamin-free Casamino acids were replaced by mixtures of known amino acids. Each amino acid was used in a concentration of 0.004 % (w/v). The amino acids were purchased from Schwartz Laboratories Inc., Mt. Vernon, N.Y. The purity of the amino acids was ascertained chromatographically.

The conditions of cultivation, count of colony formers, u.v. irradiation were as described previously (Ben-Gurion & Hertman, 1958).

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 (*Pasteurella pseudotuberculosis*). 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. (A.U./ml.).

Pesticin biosynthesis. In the general procedure for observing pesticin synthesis *Pasteurella pestis* was grown in Pr3 broth for 18 hr. at 27° until a concentration of 10⁷ bacteria/ml. was reached. The growing organisms were washed twice, diluted in phosphate buffer to a concentration of about 10⁸ organisms/ml. and u.v. irradiated for 60 sec. Samples of the irradiated bacterial suspension were diluted 1/5 in the amino acid media and incubated for 4 hr. at the desired temperature. Pesticin titrations and bacterial counts were performed during and after the incubation period. In experiments in which both bacterial growth and pesticin synthesis were determined only freshly harvested organisms were used. In experiments where only pesticin synthesis was determined the bacterial suspension could be stored at 5° for 2 days.

RESULTS

Pesticin synthesis in a complete amino acid medium

Preliminary experiments showed that the CHMG medium was as suitable as Pr3 broth for pesticin production by irradiated bacteria. When the vitamin-free Casamino acids were replaced by a mixture of 18 amino acids (L-aspartic acid, L-proline, L-lysine, L-threonine, L-glutamic acid, L-cysteine hydrochloride, L-alanine, L-serine, L-tryptophan, L-histidine, L-methionine, L-valine, L-arginine, L-leucine, L-isoleucine, glycine, L-phenylalanine and L-tyrosine) pesticin synthesis proceeded to the same extent (pesticin yield 750 A.U./ml.) as in the Pr3 broth. No quantitative differences were found in pesticin synthesis at 27° and 37°. This medium was, therefore, called the complete amino acid medium (CAA).

To determine which amino acids are essential for pesticin synthesis, 18 media

were made in each of which one of the above amino acids was omitted from the CAA medium. Irradiated bacteria were transferred to these media as well as to the CAA medium, and pesticin synthesis at 27° and 37° measured. The results are presented in Table 1. The omission of methionine, phenylalanine, valine or glycine inhibited pesticin synthesis. Leucine and isoleucine were required at 37° but not at 27°.

Table 1. *Pesticin biosynthesis in CAA medium from which one of the 18 amino acids was omitted*

Samples of 10⁸ *Pasteurella pestis* organisms/ml. were diluted 1/5 in 19 amino acid media. Pesticin synthesis after incubation at 27° and 37° for 4 hr. was measured by pesticin titration.

The amino acid omitted from CAA medium	Incubation temperature	
	27°	37°
	Pesticin concn. (arbitrary units/ml.)	
None (CAA medium)	500	500
L-Methionine	2	2
Glycine	10	10
L-Phenylalanine	10	10
L-Valine	10	2
L-Lysine	500	500
L-Leucine	500	10
L-Isoleucine	500	2

Omission of L-aspartic acid, L-proline, L-glutamic acid, L-cysteine (HCl), L-serine, L-histidine, L-threonine, L-tyrosine, L-arginine, L-alanine and L-tryptophan, did not decrease the pesticin yield at either temperature.

Minimal amino acid medium for pesticin synthesis

To determine the minimal amino acid requirement for pesticin synthesis a medium was developed containing the six amino acids found above to be essential (methionine, valine, phenylalanine, glycine, leucine and isoleucine). This medium did not support pesticin synthesis, but on addition of lysine and arginine synthesis did take place. The eight amino acids methionine, phenylalanine, glycine, valine, leucine, isoleucine, lysine and arginine together were thus found to be essential for pesticin synthesis. The medium containing these amino acids was called the minimal amino acids medium (MAA). Arginine could be omitted at 27° without decreasing the yield of pesticin. Cysteine added to MAA medium doubled the pesticin production. Addition of alanine increased the pesticin yield in MAA medium at 27°. The yield of pesticin in MAA medium was 10 % of the yield in the CAA medium containing the 18 amino acids which are found in casein hydrolysate. Quantitative differences were found between pesticin production at 27° and 37°. The yield was always considerably lower at 37° than at 27°. The omission of phenylalanine, lysine, glycine, valine, leucine, isoleucine or methionine from the cysteine-supplemented MAA medium inhibited pesticin synthesis at either temperature. The

results of these experiments are presented graphically in Fig. 1. When phenylalanine, valine and methionine were replaced by their D-isomers in MAA medium, no pestacin synthesis was observed.

The role of glucose in pestacin synthesis

Experiments were performed to establish whether the addition of glucose was essential for pestacin synthesis. Glucose-depleted bacteria cells were prepared by growing *Pasteurella pestis* for 48 hr. at 27° in glucose-free casein hydrolysate medium (CHM). These organisms were seeded again into CHM medium and incubated for 18 hr. at 27°. The actively growing organisms were harvested, washed, and pestacin synthesis in CAA medium and in cysteine-supplemented MAA medium was measured. Pestacin synthesis proceeded in the absence of glucose to the same extent as in the glucose containing controls (Table 2).

Fig. 1. Minimal amino acid requirements for pestacin biosynthesis by u.v.-irradiated *Pasteurella pestis* cells at 27° and 37°. Each column represents the average of ten experiments. The omission of L-lysine, glycine, L-valine, L-leucine, L-isoleucine, L-phenylalanine or L-methionine from the cysteine-supplemented MAA medium inhibited pestacin synthesis at either temperature.

L-Valine	+	+	+	+	+	+	+
L-Phenylalanine	+	+	+	+	+	+	+
L-Arginine	+	+	+	+	+	-	+
L-Methionine	+	+	+	+	-	+	-
L-Lysine	+	+	+	+	+	+	+
L-Leucine	+	+	+	+	+	+	+
L-Isoleucine	+	+	+	+	+	+	+
L-Alanine	-	-	+	+	+	-	-
L-Cysteine	-	+	+	-	-	+	+
Glycine	+	+	+	+	+	+	+

Amino acid requirement for growth of Pasteurella pestis, Kimberley strain P

Pasteurella pestis was grown on CHMG slants (CHMG plus 2% (w/v) agar) at 27° for 24 hr. The bacteria were harvested and washed twice with phosphate buffer. Growth in amino acid media was determined by inoculating *P. pestis*

into 3 ml. of medium in duplicate test tubes. The final concentration of the inoculum was 10^6 bacteria/ml. The tubes were incubated on a shaker for 72 hr. at 27° and growth was measured by count of colony formers. Growth of *P. pestis* was observed in the presence of six amino acids (methionine, phenylalanine, valine, isoleucine, cysteine and glycine). This medium did not support pesticin synthesis. Growth of *P. pestis* was observed also in the MAA medium. Addition of cysteine to this medium stimulated the bacterial growth (Table 3).

Table 2. *Pesticin biosynthesis in amino acid media with and without glucose addition*

Samples of 10^8 u.v.-irradiated *Pasteurella pestis* organisms/ml. were diluted 1/5 in the complete amino acid (CAA) medium and in the L-cysteine-supplemented minimal amino acid medium (CMAA), with and without 0.2 % (u/v) glucose. Pesticin synthesis was measured by pesticin titration after the 4 hr. incubation period. At zero time, no pesticin was detected.

Medium	Presence or absence of 0.2 % glucose	Incubation temperature (°C)	Pesticin conc. (arbitrary units/ml.)
CAA	+	27	500
CAA	—	27	750
CAA	+	37	500
CAA	—	37	500
CMAA	+	27	150
CMAA	—	27	100
CMAA	+	37	50
CMAA	—	37	50

Table 3. *Amino acid requirement for growth of Pasteurella pestis Kimberley strain P*

The organisms were inoculated on a basal amino acid medium containing the following amino acids: L-phenylalanine, L-valine, L-isoleucine and L-methionine. To this medium amino acids were added as indicated. Final concentration of the inoculum was 9×10^5 bacteria/ml. No growth was observed upon single addition of L-leucine, L-lysine, L-arginine or L-cysteine to the basal amino acid medium.

Amino acids added to basal amino-acid medium	Bacteria/ml. (after 72 hr.)
L-cysteine, glycine	4.1×10^7
L-leucine, L-lysine L-arginine, glycine: (MAA medium)	6.4×10^7
L-leucine, L-lysine, L-arginine, glycine, L-cysteine (CMAA medium)	1×10^8

Bacterial growth and pesticin synthesis

Freshly harvested and washed organisms in the logarithmic phase of growth were diluted to a concentration of about 10^8 bacteria/ml. and the suspension divided into two parts. One part was immediately suspended in an amino acid medium, and the growth was followed by colony count during incubation at 27° for 4 hr. The other part of the suspension was u.v.-irradiated and then suspended in the same medium; growth and pesticin synthesis were followed

simultaneously during incubation at 27° for 4 hr. A typical experiment comparing growth and pesticin synthesis in CAA and MAA media is presented in Fig. 2. The rate of growth of non-irradiated bacteria was higher in the CAA medium than in the MAA medium. The rate of pesticin synthesis by irradiated bacteria was also higher in the CAA medium. The bacteria which survived irradiation grew more slowly in CAA medium than those which were not irradiated; in MAA medium the difference was not appreciable. In order to establish whether the bacteria irradiated in the stationary phase of growth are able to synthesize pesticin a culture of *Pasteurella pestis* was harvested after

Fig. 2. Bacterial growth and pesticin biosynthesis by *Pasteurella pestis*, strain Kimberley P, in the logarithmic phase of growth in CAA and MAA medium at 27° and 37°. A, growth of non-irradiated bacteria in CAA medium; B, growth of non-irradiated bacteria in MAA medium; C, growth of irradiated bacteria in CAA medium; D, growth of irradiated bacteria in MAA medium; E, pesticin biosynthesis in CAA medium; F, pesticin biosynthesis in MAA medium.

incubation for 3 days at 27°. Growth of the non-irradiated bacteria and growth and pesticin synthesis of the irradiated bacteria were measured. Though no growth of non-irradiated bacteria was detected by colony count in CAA medium, pesticin synthesis proceeded normally in the CAA and MAA media. A typical experiment is presented in Fig. 3.

The effect of chloramphenicol on pesticin synthesis

The effect of chloramphenicol, an inhibitor of protein synthesis (Gale & Folkes, 1953; Wisseman, Smadel, Hahn & Happs, 1954), on pesticin synthesis was tested. Irradiated *Pasteurella pestis* in the logarithmic phase of growth was suspended in CAA medium containing increasing concentrations of Synthomycin U.S.P. (trade name for chloramphenicol; Abic, Ramat-Gan, Israel) and pesticin synthesis was measured after incubation at 27° and 37° for 4 hr. The effect of the same concentrations of chloramphenicol (which was

Fig. 3. Bacterial growth and pesticin biosynthesis by *Pasteurella pestis*, strain Kimberley P at the end of the logarithmic phase of growth in CAA and MAA medium at 27° and 37°. A, growth of non-irradiated organisms in CAA medium; B, growth of irradiated organisms in CAA medium; C, pesticin biosynthesis in CAA medium; D, pesticin biosynthesis in MAA medium.

below the concentration which caused bacteriostasis) on the growth of non-irradiated bacteria during the same incubation period at 27° and 37° was determined. Control measurements of growth and pesticin synthesis in CAA medium were performed. The results are shown in Table 4. It can be seen that similar concentrations of chloramphenicol inhibited both the growth of non-irradiated bacteria and pesticin synthesis by the irradiated bacteria. Chloramphenicol

at 1 $\mu\text{g./ml.}$ exerted a markedly inhibitory effect on pesticin synthesis at 27° and 37°.

Table 4. Comparison of inhibitory effect of graded low chloramphenicol concentrations on: (a) pesticin biosynthesis by irradiated *Pasteurella pestis*; (b) the growth of non-irradiated bacteria

Samples of u.v.-irradiated *P. pestis* 10^8 bacteria/ml. were diluted 1/5 in CAA medium containing low chloramphenicol concentrations and pesticin biosynthesis after 4 hr. incubation was measured. Growth of non-irradiated bacteria at the same chloramphenicol concentrations was determined by colony count. CAA medium without chloramphenicol was used for control determination of pesticin yield and bacterial growth.

Chloramphenicol ($\mu\text{g./ml.}$)	Irradiated <i>P. pestis</i> Incubation temperature		Non-irradiated <i>P. pestis</i> Incubation temperature	
	27°	37°	27°	37°
	Pesticin yield (% of control)		Bacterial growth (% of control)	
0.0	100	100	100	100
0.5	100	100	100	100
1.0	44	33	100	83
2.0	25	7	74	46
3.0	10	0	47	36

Non-pesticinogenic mutants of Pasteurella pestis

To test whether non-pesticinogenic mutants of the Kimberley strain of *Pasteurella pestis* could be obtained, the following experiments were performed. *P. pestis* (streptomycin-resistant) grown in Pr3 broth to a concentration of 10^8 bacteria/ml. were u.v.-irradiated for 5 min. This dose killed 95 % of the organisms. The irradiated bacteria were plated on Pr3A plates and tested for pesticinogenic properties by the double layer method (Ben-Gurion & Hertman, 1958). Streptomycin-sensitive *P. pseudotuberculosis* was used as indicator. Colonies which did not produce pesticin were removed with a bacteriological loop and streaked on BAB medium containing 500 $\mu\text{g.}$ streptomycin/ml. The developed colonies were streaked once more on streptomycin-containing BAB medium. The strains thus obtained were tested for rhamnose fermentation, pesticin production and pesticin sensitivity. Three independent mutants were isolated by plating 10^5 bacteria. These mutants were streptomycin-resistant and did not ferment rhamnose and were thus distinguished from the *P. pseudotuberculosis* indicator strain. These non-pesticinogenic mutants were sensitive to pesticin.

DISCUSSION

A defined amino acid medium has been developed which supports pesticin production by u.v.-irradiated *Pasteurella pestis*. In view of the requirement for amino acids and the sensitivity of the system to chloramphenicol, bacteriocinogenesis by *P. pestis* may represent a *de novo* protein synthesis.

Experiments thus far have not revealed whether the described amino acid requirements are specific for the synthesis of pesticin or represent general requirements for protein synthesis by the bacterial cell. A higher yield of pesticin was obtained in the CAA and CMAA media than in the MAA medium.

The former media also supported a higher rate of growth of non-irradiated bacteria. These observations indicate that the amino acids may be required for the general synthetic activity of the bacteria and only indirectly influence pesticin synthesis. Further proof of the dependence of pesticin synthesis upon the general synthesizing capacity of the bacterial cell may be found in the observation that maximum pesticin production occurred in a medium which supported abundant bacterial growth from a small inoculum and which thus could replace more complex media, as reported by other authors (Rockenmacher, James & Elberg, 1952). On the other hand, it may be concluded that the amino acid requirements described in this work are specific directly for pesticin synthesis, since the latter was observed also in bacteria in the stationary phase of growth. Also, *Pasteurella pestis* can be grown in a defined medium containing six amino acids (phenylalanine, valine, methionine, isoleucine, cysteine and glycine), which does not support pesticin synthesis. No decision can yet be reached between these two alternatives. Media in which maximum pesticin synthesis was not reached gave a lower production at 37° than at 27°. These observations indicate more efficient protein synthesis at 27° than at 37°. The amino acids supplied to the defined media were an adequate source of energy, and no addition of glucose was needed. Mutants of *P. pestis* which lost the pesticinogenic property became sensitive to the pesticin they originally produced. A similar behaviour has been observed for the colicinogenic strains of *Escherichia coli* (Fredericq, 1948).

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