BACTERIOCINOGENY IN PROTEUS VULGARIS

by

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SUMMARY

One hundred and eighteen different strains of Proteus vulgaris were investigated for bacteriocinogeny. These P. vulgaris strains were also used as indicators. Seventy of the 118 strains produced zones of inhibition when cross-streaked with the other P. vulgaris strains. Sixty-seven of the strains had a non-transmissible killing effect on one or more of the indicator organisms and subcultures of areas of inhibition to broth also failed to show growth. Thirty of the 67 bacteriocins with different spectra of activity were further investigated. Individual bacteriocins killed from five to 87 of the P. vulgaris indicators. Nine bacteriocins had similar host ranges whereas the host ranges of the remaining 58 bacteriocins differed. All these strains as well as the non-bacteriocinogenic Proteus strains displayed a Dienes demarcation line between their swarms. When tested against a number of gram-negative bacteria the bacteriocins only inhibited P. vulgaris and P. mirabilis strains and had no effect on strains of the family Enterobacteriaceae.

Broth cultures of bacteriocinogenic strains are inducible by ultraviolet light and yield bacteriocin titres of about 1/100. Activity can be concentrated with 40% ammonium sulphate and is sedimentable by high speed centrifugation. The bacteriocins were electrophoretically immobile and diffused through agar. Activity could be destroyed after 20 min. at a temperature of 60^{o} and also by the action

of trypsin / .....

of trypsin. Chemical analysis showed the bacteriocins to consist of protein and to contain no DNA.

Electron microscopy of all 30 preparations revealed similar phage tail-like structures with a contractile sheath round a hollow core. The particles resemble some pyocins and also the tail of a Proteus vulgaris transducing phage. In two preparations a few phage-like particles which resemble other Proteus phages were also seen. Bacteriocin activity was always associated with uncontracted sheaths and triggered tails do not adsorb to susceptible organisms. It is concluded that the tail-like structures are the products of defective lysogeny. The high incidence of defective lysogeny may be accounted for by the selection of genes which impart a selective advantage to the host and which were originally acquired through transduction or lysogenic conversion.

SAMEVATTING

Een honderd-en-agtien verskillende rasse van Proteus vulgaris is ondersoek vir die produksie van bakteriosiene. Hierdie P. vulgaris-rasse is ook gebruik as indikator-organismes. Sewentig van die 118 rasse het inhibisie-areas opgelewer na dwarsstreping met die ander P. vulgaris-rasse. Sewe-en-sestig van die rasse het 'n nie-oordraagbare dodende effek op een of meer van die indikatororganismes gehad en subkulture van inhibisie-areas het ook geen groei in boeljon getoon nie. Sewe-en-sestig van die bakteriosiene met verskillende aktiwiteitspektrums is verder ondersoek. Individuele bakteriosiene het van vyf tot 87 van die P. vulgaris- inkikators gedood. Nege bakteriosiene het eenderse gasheerreekse gehad, terwyl die gasheerreekse van die oorblywende 58 bakteriosiene van mekaar verskil het. Al hierdie rasse sowel as die niebakteriosinogeniese Proteus-stamme het 'n „Dieneslyn" vertoon tussen hul swerms. Toe die bakteriosiene teen 'n aantal gramnegatiewe bakterieë getoets is, het die bakteriosiene slegs rasse van P. vulgaris en P. mirabilis geïnhibeer en geen inhiberende effek op rasse van die familie Enterobacteriaceae getoon nie

Boeljon-kulture van bakteriosienproduserende rasse is induseerbaar deur middel van ultraviolet lig en lewer bakteriosientiters van ongeveer 1/100. Aktiwiteit kon gekonsentreer word met 40% ammoniumsulfaat, en was sedimenteerbaar met hoëspoed-

sentrifugering/.....

sentrifugering. Die bakteriosiene was elektroforeties onbeweeglik, kon deur agar diffundeer, en onderwerp aan 'n temperatuur van 60° is hulle na 20 minute vernietig. Chemiese ontleding het getoon dat die bakteriosiene uit proteïnes bestaan en geen DNA bevat nie.

Elektronmikroskopie van al 30 preparate het eenderse faag-stertagtige strukture met saamtrekbare skede rondom 'n hol kern, aan die lig gebring. Die deeltjies lyk soos sommige piosiene en ook soos die stert van 'n Proteus vulgaris-transduserende faag. In twee van die preparate is ook 'n paar faagagtige deeltjies waargeneem wat soos ander Proteus-fage lyk. Bakteriosien-aktiwiteit was altyd geassosieer met nie-saamgetrekte skedes. Saamgetrekte sterte adsorbeer nie aan gevoelige organismes nie. Die slotsom is dat die stertagtige strukture die produkte is van defektiewe lisogenie. Die hoë voorkomssyfer van defektiewe lisogenie mag moontlik toegeskryf word aan die seleksie van gene wat die gasheer begunstig en wat oorspronklik verkry is deur transduksie of lisogeniese omskepping.

CHAPTER I

REVIEW OF LITERATURE

CHAPTER I

REVIEW OF LITERATURE

INTRODUCTION

One of the first bacteria studied for its antibacterial properties was Pseudomonas aeruginosa (Emmerich & Löw, 1899. See Topley & Wilson, 1964). The antibacterial substances found were named pyocyanase (Emmerich & Löw, 1899. See Topley & Wilson, 1964), pyocyanin (Ehrismann, 1934), and α-hydroxy-phenazine (Schoental, 1941), and are active against a large variety of gram-positive and gram-negative bacilli.

Gratia in 1925 observed inhibition of Escherichia coli ∅ by E. coli V (Gratia, 1925). The inhibitory substance, to which the name colicin was later given by Gratia and Fredericq in 1946, diffuses through agar and through cellophane membranes, can be precipitated with acetone, and is resistant to heat and the action of chloroform (Adams, 1959). Jacob et al. (1953) defined the term bacteriocin as a protein-like substance, the biosynthesis of which is lethal for the producing organism and no multiplication of the bacteriocin occurs. Most bacteriocins act only on certain strains

related / .....

related to the producer species although some bacteriocins also act on a limited number of strains of related species. The action of bacteriocins is dependent on the presence of specific receptors on the surface of susceptible cells. Bacteriophage-like structures which kill bacteria but do not multiply, comply with this operational definition and in recent years such objects liberated by bacteria have been linked with bacteriocinogeny.

Antagonism between one species and another and even between members of the same species occurs throughout nature, and the term 'antibiosis' which means, literally, 'against life', was introduced by Vuillemin in 1889 (Barber & Garrod, 1963). The term antibiotic is generally used to mean an antibacterial substance derived from a living source.

Nomenclature

A derivation of the Linnaean specific name of the producing organism is given to its bacteriocin, e.g. the bacteriocins of Escherichia coli and Pseudomonas pyocyanea are called colicins and pyocins respectively (Bradley & Dewar, 1966).

Distribution / .....

Distribution of bacteriocinogenic strains.

The bacteriocins were recognised as being different from antibiotics and as more bacteriocins were discovered they were accepted as a distinct class of biological compounds

Colicins were the first bacteriocins to be studied in detail. Fredericq attempted to group the colicins according to their spectrum of activity on wild strains of Escherichia coli and also on mutants resistant to one or other colicin and demonstrated that colicins adsorb to specific receptor sites on the cell wall. With the use of these criteria he grouped colicins into 17 types (Fredericq, 1948). Hamon & Peron (1963a) refer to 23 types. The 17 types of Fredericq are A, B, C, D, E, F, G, H, I, J, K, V, S_1, S_2, S_3, S_4, and S_5. This classification is further complicated by frequent appearances of strains which produce more than one colicin and of mutants resistant to more than one colicin type. Thus the six colicin types E, F, J, S_2, S_3, and S_5, were later grouped into a new type E by the judicious use of indicator strains. Within each species there is also a tendency to produce only certain colicin types. Thus E. freundii strains only produce type A; E. coli strains produce types B, C, D, E, F, G, H, I or V; J and K are produced by Paracolobactrum spp; Shigella spp. produce S_1, S_2, S_3, S_4, and S_5 while I, K or B are produced by Salmonella strains (Reeves, 1965).

The alveicins / .....

The alveicins, arizonacins, pneumocins and aerocins which are produced by Hafnia spp., Paracolobactrum arizonae, Klebsiella spp. and Aerobacter aerogenes respectively, were discovered by Hamon & Peron (1963a). The alveicins act on Hafnia spp., E coli strains and some Shigella paradysenteriae strains while the arizonacins show activity on some strains of P. arizonae and E. coli. The activity of the pneumocins is generally limited to the other strains of Klebsiella and no activity can be found on any of the colicin indicators but occasionally on strains of Aerobacter aerogenes. Enterobacter cloacae and Klebsiella are the only strains except for A. aerogenes on which the aerocins act.

The caratovoricins, produced by strains of Erwinia spp. also act on E. coli strains as well as on strains of Pseudomonas fluorescens, on two Serratia spp. and on Xanthomonas spp. and have a range of activity on strains of E. coli, Xanthomonas and Erwinia (Hamon & Peron, 1961a). The study of the bacteriocins of Serratia spp. - the marcescins - show them to be bactericidal for strains of Serratia, E. coli and Erwinia, and it appears from this work on marcescins that Serratia strains can produce two types of bacteriocins, one that is similar to the colicins and one that can only be classified as a marcescin (Hamon & Peron, 1961b).

Strains of Pasteurella pestis are found to produce an anti-

biotic / .....

biotic which is active on strains of P. pseudotuberculosis (Ben-Gurion & Hertman, 1958). This antibiotic is named pesticin I. Brubaker & Surgalla (1961) discovered a second pesticin (II) which is produced by all the strains of P. pseudotuberculosis and P. pestis tested. There exists a close antigenic relationship between P. pseudotuberculosis type II and the Salmonella B group, and between P. pseudotuberculosis type IV and the Salmonella D group. Knapp (1960) suggested that this antigenic relationship between these two organisms lends support to the idea that P. pseudotuberculosis may be related to the Enterobacteriaceae. A P. pestis phage which had been adapted to P. pseudotuberculosis was also found to lyse certain strains of Salmonella and Shigella. This fact also suggests (Lazarus & Gunnison, 1947) an antigenic relationship between strains of Salmonella and Shigella and P. pseudotuberculosis as the action of bacteriophages is limited to closely related species.

Hamon, Veron & Peron (1961b) discovered a bacteriocin produced by Pseudomonas fluorescens namely fluocin. All the strains which produce fluocins have different activity spectra on the P. fluorescens indicators. Only one of the fluocins is bactericidal for any of the strains of P. pyocyanea tested.

Bacillus megaterium strains produce bacteriocins which are named megacins (Ivanovics & Nagy, 1958). Only some B. anthracis

and / .....

and B. subtilis strains are sensitive to the megacins. Holland (1963) described some of the properties of a new bacteriocin, megacin C, which several strains of B. megaterium produce.

Bacteriocins active against several cultures of the same species are produced by Listeria monocytogenes; these bacteriocins are named monocins and can be classified into types A and B on the basis of cross-resistance. The monocins have no effect on the Gram-negative bacteria or on strains of Streptococcus but they do act on strains of Staphylococcus and Bacillus (Hamon & Peron, 1962).

The first cerecin was discovered by McCloy (1951) in a study of Bacillus phages. They are produced by strains of Bacillus cereus and do not act on any known indicator strains for the bacteriocins of Gram-negative bacteria (Hamon & Peron, 1963b).

Various species of Streptococcus of the enterococcus group produce bacteriocins, namely the enterococcins (Brock, Peacher & Pierson, 1963). The enterococcins are classified into five types on the basis of their sensitivity to proteolytic enzymes, heat, chloroform, and their activity spectra. Depending on the types of enterococcin, they act on strains of S. zymogenes, S. faecalis, S. faecium and S. liquefaciens.

Fredericq (1946) was the first to study bacteriocins from

strains of Staphylococcus. Hamon & Peron (1963d), also studying the staphylococcins, discovered that, apart from the strains of Staphylococcus and Bacillus which Fredericq had found to be sensitive to these bacteriocins, strains of Listeria and Corynebacterium are also sensitive to staphylococcins.

In 1954 Jacob described a new antibacterial principle produced by strain 10 of Pseudomonas pyocyanea which differed in its properties from the other known antibiotics such as pyocyanin (Ehrismann, 1934) and pyocyanase (Emmerich & Löw, 1899. See Topley & Wilson, 1964). This substance, which he named pyocin, is mainly active on other strains of P. pyocyanea and has a similar specific bactericidal activity typical of bacteriocins.

Cradock-Watson (1965) discovered bacteriocins in strains of Proteus hauseri. These bacteriocins are only active on P. hauseri strains and do not inhibit any other strains of the family Enterobacteriaceae. Coetzee (1967) described morganicins, produced by P. morganii, as well as bacteriocins produced by Providence strains. No extraspecies activity can be demonstrated for the morganicins or the bacteriocins produced by Providence, except for Providence strain NCTC 9190 which has an inhibitory effect on a P. rettgeri strain.

Strains of / .....

Strains of Alcaligenes faecalis are found to produce bacteriocins (Mar'e & Coetzee, 1964). They are inhibitory for several strains of A. faecalis as well as strains of Escherichia, Shigella, Serratia, Staphylococci and Proteus.

De Klerk & Coetzee (1961) discovered that one heterofermentative and eleven homofermentative strains of the family Lactobacteriaceae produce bacteriocins. The action of these bacteriocins is found to be restricted to certain members of the same family.

Ryan, Fried & Mukai (1955) and Mukai (1960) reported that upon irradiation with ultraviolet light, strain 15 of Escherichia coli would lyse and release an antibacterial agent. Since no bacteriophages were detected in the lysate, they considered the agent to be a colicin. In order to further elucidate the properties of colicin 15, Endo et al. (1965); Sandoval, Reilly & Tandler (1965) and Mennigmann (1965a) found that the bactericidal activity could be concentrated by high speed centrifugation. Electron microscopy of this fraction showed it to consist of small-headed phage-like particles, and the quantity of phage-like bodies seems to correlate with the degree of biological activity.

In an investigation of the biochemical nature of the pyocin produced by Pseudomonas aeruginosa strain R, Kageyama & Egami (1962) and Kageyama (1964) found that the pyocin appeared similar

to rod-like / .....

to rod-like particles when examined in the electron microscope, and Ishii, Nishi & Egami (1965) could demonstrate at least two structural components of this pyocin which resemble the headless contractile tails of bacteriophages.

Bradley & Dewar (1966) studied the morphology of bacteriocins with the electron microscope and ascribed the colicin H activity of Escherichia coli A 10 to phage-like particles similar to colicin 15. They also proved that three other pyocinogenic strains of P.aeruginosa liberate structures similar to those of strain R, and that a monocin liberated by a strain of Listeria monocytogenes consists of phage-like particles. Takeya et al. (1967) showed that pyocin 28 consisted of cross-striated rods about 1000Å in length and another pyocin produced by P.aeruginosa strain C_9 was described by Higerd, Baechler & Berk (1967).

In several Bacillus spp. a few phage-like particles, which are apparently physiologically identical to bacteriocins, but have not been classified as such were isolated by Seaman, Tarmy & Marmur (1964) and Stickler, Tucker & Kay (1965). These were described as defective temperate bacteriophages which are able to lyse sensitive cells but unable to multiply intracellularly.

Taubeneck (1963), while testing a number of Proteus strains and their stable L-forms for lysogeny, discovered that Proteus mirabilis / .....

bilis strain 52 liberates tail-like structures, which contract upon adsorption, and kill some P. mirabilis and P. vulgaris strains. In an investigation of microtubules in two strains of P. mirabilis, Van Iterson, Hoeniger & Nijman van Zanten (1967) demonstrated that both strains, when induced by mitomycin C, produce phage tail-like structures similar to the pyocins described by Ishii et al. (1965).

Nature of Bacteriocins

Bacteriocins comprise a group of varied antibiotic substances, which differ in numerous characteristics. Mennigmann (1965b) devised a list of different criteria to characterize antibacterial agents and to differentiate bacteriocins from bacteriophages and defective bacteriophages :-

1. Production of antibacterial agent with ultraviolet irradiation, mitomycin C treatment or thymine deprivation.
2. Antibacterial activity limited to the number of related strains.
3. Loss of antibacterial activity on heating.
4. Loss of antibacterial activity on treatment with trypsin.
5. No transmissibility of the antibacterial activity.
6. Lysis of bacterial culture on induction.

7. No / .....

7. No antibacterial activity left in the supernatant fluid after high speed centrifugation.

Numbers 1 to 3 above indicate the presence of an antibacterial agent, Nos. 4 and 5 favour the antibacterial agent as being a bacteriocin and Nos. 6 and 7 favour the antibacterial agent as being a bacteriophage or defective bacteriophage.

Fredericq (1948, 1957) used the following criteria to distinguish different colicins from each other :-

1. Extent and specificity of the activity spectrum.
2. Specificity of resistant mutants.
3. Extent of diffusibility in agar.
4. Temperature sensitivity.
5. Sensitivity to proteolytic enzymes.
6. Electrophoretic mobility.

Bacteriocinogenic factors

The ability to produce a colicin is a stable heritable property which is governed by genetic determinants called colicinogenic factors.

Fredericq (1954) discovered that some colicinogenic strains

of / .....

of Escherichia coli and Shigella, when grown in broth with non-colicinogenic strains, transmitted their colicinogenic property to the non-colicinogenic cells. He worked with E. coli K_{12}, which was colicinogenic for colicin E_1, and showed that only the F^+ strains transmit colicinogeny while no transfer of colicin E_1 occurs in crosses with F^- cells.

Alföldi, Jacob, Wollman & Mazé (1958) and Clowes (1963) were able to demonstrate that colicin E_1 was not integrated into the bacterial chromosome. Hfr strains with differing 'origines' and orientations of transfer were used as donors of colicin E_1. By interrupted mating experiments they showed that there was a constant time of entry of the colicin E_1 factor from each Hfr donor.

Ozeki & Stocker (1958) were able successfully to transmit colicin C_2 (classified as type E) to Salmonella typhimurium and Escherichia coli strains by transduction with phage PLT 22 and phage P_1. The frequency of transduction was the same as that found for transduction with other markers (Adams, 1959).

Ozeki, Stocker & Smith (1962) investigated the transmission of colicinogeny between strains of Salmonella typhimurium. They worked with non-colicinogenic S. typhimurium strain LT2 and different strains of Escherichia coli which were colicinogenic for colicin E_1, B or K and a Shigella sonnei strain which produced colicins I and E_2.

It was / .....

It was found that the five colicins are transferred from the E. coli and S. sonnei strains into the S. typhimurium strain and each colicinogenic factor causes the production of a colicin which is indistinguishable from the original colicin transferred from the donor organism. These different colicinogenic strains of S. typhimurium LT2 were mated with different non-colicinogenic strains of S. typhimurium and it was found that colicins I and B are transmitted by singly colicinogenic S. typhimurium strains whereas colicin E_2 and K are not. However strains LT2 carrying either colicin I or colicin B in addition to colicin E_2, colicin K and colicin E_1, transmit both.

The colicin I and B factors, as distinct from the other colicin factors, were found to behave like sex factors. Ozeki, Stocker & Smith (1962) found that colicin I and B promote conjugation of cells through which the transfer of non-infective colicin factors, such as colicin E_1 and E_2, occur.

The phenomenon of epidemic spread among non-colicinogenic strains was observed by Stocker, Smith & Ozeki (1963) in colicin I and B. They suggested that many or all the bacteria newly infected by either of these factors became 'effective donors' and that this property of high frequency transfer of colicinogeny was maintained by the newly converted cells for a few generations. Thereafter, only a small proportion of colicin I^+ bacteria can denote colicin I, because

the donor / .....

the donor ability becomes repressed in the same way as the function of other newly introduced structural genes, such as in λ-lysogenic cells (Pardee, Jacob & Monod, 1959).

Meynell & Lawn (1967) described a new type of sex pili in the conjugational transfer of colicin factor Ib by Salmonella typhimurium. These Ib pili differ from other known pili in that they are morphologically distinct from common pili and other sex pili such as those determined by the F factor. They do not adsorb any of the F specific phages but do adsorb the I specific phage (Lawn, Meynell, Meynell & Datta, 1967). Meynell & Lawn (1967) found that there is a correlation between the incidence of cells with Ib pili and the ability to donate colicin Ib. The donor ability was also permanently diminished by a combination of repeated high speed blending and periods for regeneration of pili. As a result of these findings, Meynell & Lawn (1967) suggest that the Ib pili play a role in the transfer of colicin Ib during conjugation.

Under the conditions of partial thymine deprivation, Clowes (1965) found it possible to eliminate colicins with high efficiency from thymineless strains as well as the elimination of F factors from F^+ cells and of RTF factors, but found no elimination of the integrated F sex factors (Hfr factors).

Except for colicins I and B, the colicinogenic factors are

plasmids / .....

plasmids which replicate in phase with the bacterial chromosome without killing the cells. When a non-colicinogenic cell becomes colicinogenic, the colicin also confers immunity to that colicinogenic cell. Nomura & Maeda (1965) suggested that the immunity is due to a change in some component or structure, perhaps in the cell membranes, which is important in the transmission of the specific stimulus which eventually affects the target in the sensitive cells.

Pasteurella pestis differs from P. pseudotuberculosis in that it contains the fibrinolytic factor (F), the coagulase factor (C) and is non-motile. Brubaker, Surgalla & Beesley (1965) noted that the production of F and C is correlated with the production of pesticin I determinant (PI). They found that the three structural genes for the three activities are linked and situated on an extrachromosomal determinant. These workers (Brubaker, Beesley & Surgalla, 1965) also observed that a non-pesticinogenic strain of P. pestis resembles that of a wild-type P. pseudotuberculosis. They suggest that mutational events take place in P. pestis to convert it to a form which resembles P. pseudotuberculosis and that conversion of P. pseudotuberculosis to a form which resembles P. pestis could conceivably occur upon donation of the PI determinant by P. pestis.

Production / .....

Production of Bacteriocins

According to Jacob et al. (1953) one of the criteria which determines a colicin is its lethal biosynthesis - colicin production involves the death of the bacterium without lysis. Although bacteriocinogenic strains possess the genetic ability to produce bacteriocin, they do not do so under all conditions.

The discovery by Jacob, Siminovitch & Wollman (1952) that colicin ML-E, produced by Escherichia coli ML, could be induced by ultraviolet light, led Fredericq (1954), and later Hamon & Lewe (1955) to use this method of induction for the production of colicins in different strains of E. coli.

The hypothesis that all the colicin produced by a colicinogenic culture, either spontaneously or after induction by ultraviolet light irradiation, was synthesised and released by a portion of the colicinogenic cells and that these cells were subsequently non-viable, led Ozeki, Stocker & Margerie (1959) to study the kinetics of colicin production. They used a Salmonella typhimurium strain which had been made colicinogenic for colicin E_2. When a mixture of the colicinogenic strain and the sensitive strain was made, and incorporated into a soft agar layer, they found that tiny clear spots were formed in the lawn of organisms. Each of the clear spots originated from a single bacterium and they proposed the term lacunae for these

inhibitory / .....

inhibitory areas. They were also able to demonstrate by micromanipulative isolation that the production of colicin was a lethal event and the cells which released the colicins were non-viable. In non-lysogenic strains of Escherichia coli, there is a continuous release of colicin. In strains that are bacteriocinogenic as well as lysogenic, the release is at the time of lysis (Reeves, 1965).

Iijima (1962) found that by adding chloramphenicol after induction, no colicin production was detected and suggested as a result of these findings that colicin production was a de novo synthesis. Ben-Gurion (1965) also showed that the addition of fluorouracil and thymine after irradiation prevented the production of colicin and suggested that this result indicated a de novo synthesis of RNA which was necessary for the production of colicin in colicinogenic cells.

Ikeda, Kageyama & Egami (1964) found that the pyocin produced by Pseudomonas aeruginosa strain R was synthesised de novo only after induction. They detected no DNA synthesis after induction and the release of the pyocin was concomitant with cell lysis.

Megacinogenic strain 216 does not normally excrete megacin (Ivanovics, 1962), but this strain proves to be high inducible when exposed to ultraviolet light irradiation. Megacin production was found not to be associated with normal multiplication of the cells

but was / .....

but was a lethal biosynthesis when induced by ultraviolet irradiation.

The production of staphylococcins could only be demonstrated on solid media. No staphylococcins could be detected in fluid medium (Lachowicz, 1965).

Hertman & Ben-Gurion (1958) found that the production of pesticin I was inducible with ultraviolet irradiation. Pesticin I was excreted into the surrounding medium without lysis of the producing organisms.

Mode of action of bacteriocins

Fredericq (1952) showed that colicin K could be adsorbed out of a solution by sensitive bacteria and Hamon & Peron (1960) were able to prove that this adsorption was specific. Their work showed that six different colicins and five different pyocins could be adsorbed by sensitive strains but not by resistant mutants. Data produced by Holland (1962) showed that megacin 216 was adsorbed to the sensitive strain Bacillus megaterium 207M.

The kinetics of killing by bacteriocins was first studied by Jacob, Siminovitch & Wollman (1952) with the use of colicin ML-E. Their results as well as those for pyocin C10 (Jacob, 1954) suggested that one bacteriocin particle killed one bacterium. Kageyama,

Ikeda & Egami (1964) were also able to confirm the results of Jacob (1954) with a pyocin produced by Pseudomonas aeruginosa strain R.

Nomura & Maeda (1965) proposed a model whereby the attachment of a single colicin particle to a receptor site on the cell wall causes an irreversible change in the receptor site. This change in the receptor site is transmitted to the sensitive target within the cell, presumably along the cell membranes, which leads to the death of the cell.

Nomura & Nakamura (1962) and Nomura (1963) found that high multiplicities of colicin K inhibits the oxidative phosphorilation system of the cell, which leads first to the inhibition of DNA synthesis and then to the inhibition of RNA and protein synthesis, as well as the inhibition of the active transport of potassium through the cell surface. They also found that the reproduction of virulent phage T_4 was inhibited when colicin K was added soon after infection but that it does not induce the development of λ in lysogenic cells.

Colicin E_2 induces the degradation of DNA in the cell and this degradation is dependent on the multiplicity of colicin E_2 (Nomura, 1963). No degradation of RNA was detected. Colicin E_2 induces the development of λ in lysogenic Escherichia coli cells, and has no effect on the oxidative phosphorilation system or on the active transport of potassium.

Colicin / .....

Colicin E_3 inhibits protein synthesis but not DNA or RNA synthesis (Nomura, 1963). The inhibition of protein synthesis by colicin E_3 was studied by Konisky & Nomura (1967) and it was found that some specific alteration of the ribosome leads to an inactivation of the specific transfer RNA binding function. Colicin E_3 does not degrade RNA, nor does it induce λ in lysogenic cells.

The inhibition of macromolecular synthesis and phage growth in both colicin K treated cells, and in colicin E_3 treated cells was reversed by the treatment with trypsin. The killing action of colicin E_2 was only poorly reversible by treatment with trypsin. This may be due to the irreversible breakdown of DNA (Nomura, 1963). Fredericq (1958) suggested that the reversal of the killing action of colicins by trypsin treatment was due to the digestion of the attached colicin particle, since colicins are sensitive to trypsin.

The pyocin produced by Pseudomonas pyocyanae C10 which was discovered and studied by Jacob (1954) caused the respiration of the bacterial suspension to decrease gradually. Bacteria that have adsorbed the pyocin, do not multiply and eventually become non-viable.

The megacins act on the cell membrane of sensitive cells (Ivanovics, Alföldi & Nagy, 1959). Within 10 to 15 minutes there is a marked drop in respiration and the cell contents start to leak out.

In the / .....

In the case of megacin C (Holland, 1963) there is a breakdown of the DNA of the sensitive cells. RNA and protein synthesis also stop, but no degradation of RNA takes place.

Studies by Puck & Lee (1955) showed that cell leakage, induced by T_2 bacteriophage in the course of normal infection, slows down within a few minutes. This effect on cell permeability produced by viable phage is reversed by a "sealing reaction" induced by the phage after infection.

Phage tail-like structures possibly act in the same way as phage ghosts. The mechanism of killing by ghosts is different from that of viable phage in that the phage ghosts lack the cell wall repair mechanism (Terzi, 1967). Phage ghosts puncture the cell wall and cause cell leakage which eventually leads to cell death (Herriot, 1951).

Chemical nature of bacteriocins

Bacteriocins are set apart from the other antibiotics by their size. Goebel & Barry (1958) and Amano, Goebel & Miller-Smidth (1958) discovered that colicin K was associated with the O somatic antigen of Escherichia coli K_{235}. The O somatic antigen is a lipocarbohydrate protein complex, and on dissociation of the complex two components were found. The first component is rich

in protein / .....

in protein and has a bactericidal activity ten times greater than the colicin itself. The second component is a lipopolysaccharide which has the properties of an endotoxin and is devoid of colicin activity.

Ribi et al. (1964) reported that extraction of gram-negative bacterial cell walls with aqueous phenol yields a toxic macromolecular complex which is named endotoxin and which consists of protein, lipid and polysaccharide. The polysaccharide of the endotoxin is the O somatic antigen of the bacterial cell wall and it was found that the protein can be eliminated from the lipopolysaccharide protein complex without loss of toxicity. It has been suggested that the toxicity of the lipopolysaccharide endotoxin resides primarily in the lipid and that the polysaccharide moiety merely functions as a carrier (Westphal & Lüderitz, 1954).

Hutton & Goebel (1962) showed that colicin V was a lipocarbohydrate complex which is associated with O antigen of the producing strain Escherichia coli K_{357}. An unidentified colicin (Nüske, Hösel, Venner & Zinner, 1957) produced by E. coli S.G. 710 was also found to contain protein lipid and carbohydrate. Moreover this colicin was similar to the O somatic antigen of the parent strain.

Chemical analysis of colicin A (Barry, Everhart, Abbott & Graham, 1965) showed it to consist mainly of protein and to contain

no DNA. / .....

no DNA. No relationship was found between colicin A and the specific O and H antigens present on the surface of strain Escherichia coli CA_{31}, which produces this colicin.

Purified colicin F (E_2), obtained from Escherichia coli CA_{42}, contains a high percentage of protein but lacks the lipid component (Reeves, 1963). It is suggested that colicin F does not form part of the O somatic antigen of the parent organism.

Keene (1966) studied the chemical analysis of colicin I produced by a strain of Escherichia coli. It was found to be a lipo-carbohydrate protein complex.

Megacin 216 (Holland, 1961) in contrast to colicin K, was isolated free from any lipocarbohydrate complex. It was found to be a chemically pure protein molecule.

Studies on the pyocin produced by Pseudomonas aeruginosa strain R (Kageyama, 1964) showed it to be a protein, which consists of 20 amino acids and a negligible amount of sugars.

De Klerk & Smit (1967) investigated the properties of a bac-teriocin produced by Lactobacillus fermenti. This bacteriocin was found to be a lipocarbohydrate protein with small amounts of hexo-samine and phosphorus. The protein fraction was found to contain 16 amino acids.

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CHAPTER II

INCIDENCE OF BACTERIOCINOGENY IN PROTEUS VULGARIS

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INTRODUCTION

The discovery by Lwoff, Siminovitch & Kjelgaard (1950) that the mutagenic agent, ultraviolet light, causes mass lysis of lysogenic cells with the concomitant production of phage particles by almost all the cells led Fredericq (1954) to use this method of induction on colicinogenic strains. Fredericq (1954) found that colicins, like bacteriophages, were inducible, but in contrast to bacteriophages did not lyse the producing strain. Under these experimental conditions only a few cells produced colicin, while the effect of ultraviolet light was to induce a large proportion of cells (about 50%) to produce colicin.

Mitomycin C, and antibiotic isolated from Streptomyces caespitosus, was found to inhibit selectively the synthesis of DNA in Escherichia coli (Shiba, Terawaki, Taguchi & Kawamata, 1959). Iijima (1962) was the first to use mitomycin C successfully on E. coli K 30 for the induction of colicin K production.

The induction / .....

The induction of bacteriocin production may also be brought about by temperature sensitive mutants of a bacteriocinogenic strain. Kohiyama & Nomura (1965) found that they could induce colicin E_2 in a temperature sensitive mutant of Escherichia coli K 12 strain 162.

Thymine deprivation also induces bacteriocin production. This first became known when Mennigmann (1964) found that a thymine auxotrophic strain of Escherichia coli, colicinogenic for colicin 15, can be induced when the cells are deprived of thymine.

The method used to indicate the presence of a bacteriocin in a bacterial culture was originally devised by Fredericq (1948). Bacteriocinogenic cultures were streaked on agar plates and overlayed with a top layer seeded with the sensitive indicator organism. This method for the detection of bacteriocins was later simplified by Abbott & Shannon (1958), and is the method used in this study for the detection of bacteriocins.

An attempt was made to demonstrate the production of bacteriocins by different strains of Proteus vulgaris on solid medium.

METHODS

Media

(a) Liquid media

1. Nutrient Broth: / .....

1. Nutrient Broth: Difco nutrient broth powder, 16 gm.; NaCl, 10 gm.; Oxoid lab-lemco broth powder, 16 gm.; Difco tryptose broth, 52 gm. Dissolve these substances in 2 litres distilled water, steam for 45 min. Add 2ml. N-$CaCl_2$ solution. Adjust the pH to 7.4 by adding 4% NaOH solution. Bottle and sterilize in the autoclave.

2. Difco Brain-heart infusion broth: Bottled in 50 ml. and 100 ml. quantities.

3. Difco MacConkey Broth.

(b) Solid media

1. Difco MacConkey agar.
2. Difco SS-agar.
3. Nutrient agar made up as follows :

(i) Meat extract: 2 lbs. minced lean meat - add 2000 ml. tapwater - leave overnight at 4°. Filter through cheesecloth. Steam 1 hr. Filter through filter paper. Steam 1 hr. Leave overnight.

(ii) Add Ocean Gold agar, 30 gm. to the 2000 ml. meat extract, Steam until dissolved. Add Difco Peptone, 20 gm.; NaCl, 10 gm. Adjust pH to 7.4 by adding 4% NaOH solution. Add 14 ml. of a 3.5% Na_2CO_3 solution. Steam for $\frac{1}{2}$ hr. Filter through cheesecloth. Autoclave.

Bacterial Cultures

One hundred and eighteen strains of Proteus vulgaris were locally isolated during 1966 and investigated for bacteriocinogeny. Nine bacteriocinogenic strains were selected and tested against a variety of gram-negative organisms (Table I).

Strains were maintained at 4°. Cultures were incubated at 25°.

TABLE I

	No. indicator strains
Proteus mirabilis	44
P. morganii	13
P. rettgeri	15
Providence	9
Escherichia coli	18
Salmonella spp.	6
Salmonella typhosa	2
Shigella spp.	14
Alcaligenes faecalis	7
Serratia marcescens	7

Table I. Various gram-negative organisms used as indicator strains for nine bacteriocinogenic strains selected at random.

Detection / .....

Detection

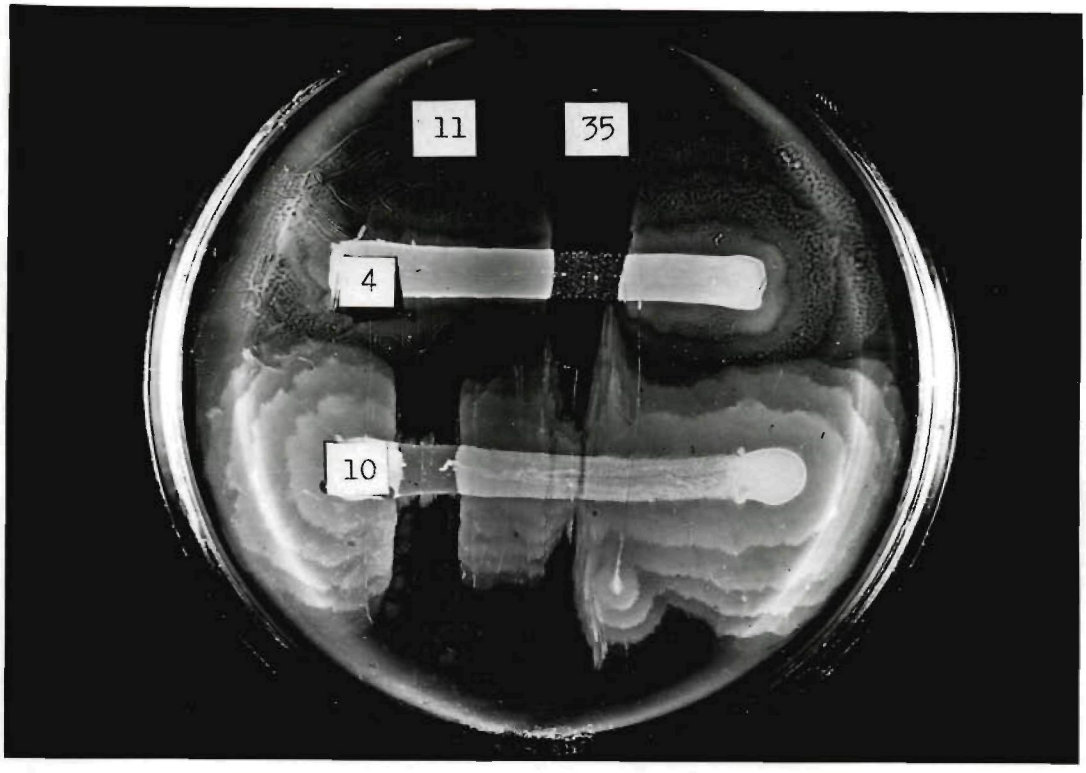
A modification of the method of Abbott & Shannon (1958) was used. In preparation for the test for bacteriocinogenic activity, each of the 118 P. vulgaris strains was inoculated in nutrient broth and incubated overnight. Each culture was then inoculated across an SS-agar plate to give a confluent streak of growth. The plates were incubated for 7 hr. The organisms on the plates were killed by exposure to chloroform. For this purpose a disc of filter paper was placed in the lid of a Petri dish and saturated with chloroform. The portion containing the medium was replaced and the plate left inverted for $\frac{1}{2}$ hr. The growth was scraped to one end with the edge of a clean slide and then removed together with a small portion of agar. Overnight broth cultures of the organisms to be tested for sensitivity to the bacteriocins were then inoculated across the plate at right angles to the position formerly occupied by the primary streak. The plates were incubated for 16 hr. (Plate I).

Ultraviolet light irradiation

The method of detection of bacteriocins as described above was repeated with plates that were duplicates of those used in the foregoing experiment. These plates were irradiated for 4 min. with the use of a 30-W Hanovia sterilamp (wavelength 2537 Å) from a distance of 25 cm. The plates were incubated for 16 hr. in the

dark / .....

PLATE I



Proteus vulgaris strains 11 and 35 cross-streaked according to the method of Abbott & Shannon (1958) with P. vulgaris indicator organisms 4 and 10. Areas of inhibition in the confluent growth of the indicator organisms indicate sensitivity to bacteriocin.

dark to prevent photoreactivation (Kelner, 1949; Newcombe, 1955) and thereafter treated in the same way as described above.

Transmissibility

Serial transmissibility of the killing effect was tested by cutting out a small piece of the area of inhibition with a sterile wire loop. This was then transferred to broth. The broth was sterilized with a few drops of chloroform. After the chloroform had been bubbled off, dilutions of the suspension were spotted on a lawn of the indicator organism. Nine strains of Proteus vulgaris which produce bacteriocins were selected and treated in the same way as described above, except that the suspensions were spotted on lawns of different organisms of the family Enterobacteriaceae.

The bactericidal property of the bacteriocins was tested by subculture in broth of clear areas of inhibition.

RESULTS

Incidence

Seventy of the strains produced areas of inhibition on one or more of the Proteus vulgaris cross-streaks. These bacteriocins inhibited from five to 87 P. vulgaris indicators. Nine bacteriocins

(Nos. 11, 31, 48, 53, 54, 66, 95, 97, 117) have similar host ranges. The host ranges of the remaining 58 bacteriocins differ (Table II).

Only three (Nos. 10, 27, 116) of the seventy inhibitory areas were transmissible and formed plaques on the indicator organisms. The bacteriocins killed sensitive organisms, since the subcultures of clear areas of inhibition failed to show growth.

None of the nine bacteriocins tested inhibited Proteus morganii, P. rettgeri or non-Proteus species, but they all inhibited 4 to all 44 P. mirabilis strains (Table III).

Ultraviolet irradiation

All the bacteriocinogenic strains were tested for inducibility of bacteriocins on agar plates. The bacteriocinogenic cultures were found to produce more distinct areas of inhibition on agar with ultraviolet light irradiation. As a result of this finding all the cultures were irradiated to increase bacteriocin production.

DISCUSSION

All the bacteriocinogenic strains were found to be inducible. This is analogous to the results of Jacob (1954) for the pyocin produced

TABLE II: 118 *PROTEUS VULGARIS* STRAINS CROSS-STREAKED AGAINST EACH OTHER FOR THE DETECTION OF BACTERIOCINOGENY:

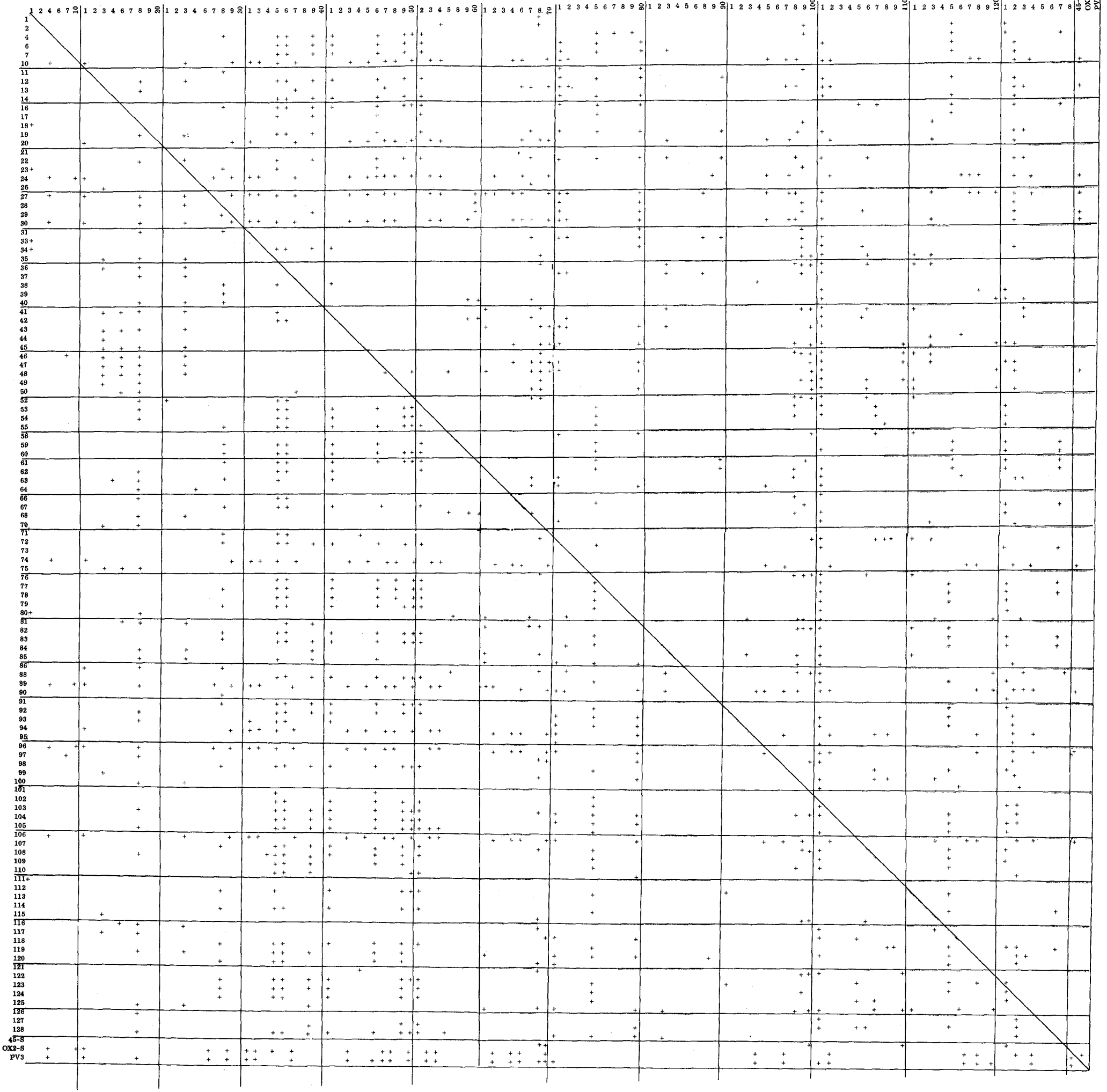
+ INDICATES BACTERIOCIN PRODUCTION;

BLANK INDICATES NO PRODUCTION OF BACTERIOCIN.

TABLE II

BACTERIOCIN PRODUCING STRAINS

BACTERIOCIN SENSITIVE STRAINS



T A B L E III

P.mirabilis organisms	Bacteriocin No. 11	35	41	49	50	70	71	80	116
15a	-	+	-	+	+	-	-	+	-
$F10_8$	-	-	-	+	+	-	-	+	+
13c	-	-	-	+	+	-	+	+	+
193	-	-	-	+	+	-	-	+	+
P.V.8	-	-	-	-	+	-	-	+	-
OXK	-	+	+	+	+	-	-	+	-
$10j/s_2$	-	-	-	-	+	-	-	+	-
P.V.44	-	-	-	-	+	-	-	+	-
61	-	-	-	-	+	-	-	+	-
113	-	+	+	+	+	-	-	+	-
58	+	-	-	-	+	+	-	+	-
78	-	-	-	-	+	-	-	+	-
12	-	+	+	+	+	-	-	+	-
SAIMR 8	-	+	+	+	+	-	+	+	-
P.V.11	-	-	-	-	+	-	-	+	+
$OX2/s_6$	+	-	-	+	+	+	+	+	+
P.V.12	-	+	+	+	+	-	-	+	-
P.V.1	-	-	-	-	+	-	+	+	-
P.V.36	-	-	-	-	+	-	-	+	-
$P.V.9/s_1$	+	-	-	+	+	+	-	+	+
74	-	-	-	-	+	-	-	+	-
P.V.30	-	-	-	-	+	-	-	+	-
65	-	+	+	+	+	-	-	+	-
48	-	+	+	+	+	-	+	+	-
171	-	+	+	+	+	-	-	+	-
121	-	-	-	-	+	-	-	+	-
54	-	-	-	-	+	-	-	+	-

Continued / .....

Continued / .....

P. mirabilis organisms	Bacteriocin No. 11	35	41	49	50	70	71	80	116
78	-	-	-	-	+	-	-	+	-
176	-	-	-	-	+	-	+	+	-
67	-	+	+	+	+	-	+	+	-
170	+	+	+	+	+	-	-	+	+
23	-	-	-	-	+	-	-	+	-
182	+	+	+	+	+	-	-	+	+
14	-	-	-	-	+	-	-	+	-
51	-	-	-	-	+	-	-	+	-
192	-	-	-	-	+	-	-	+	-
24	+	-	-	-	+	-	-	+	+
59	-	-	-	-	+	-	-	+	-
63	-	-	-	-	+	+	-	+	-
20	-	-	-	-	+	-	-	+	-
193	+	-	-	-	+	-	-	+	+
57	-	-	-	-	+	-	-	+	-
9	-	+	+	+	+	-	-	+	-

Table III. Nine bacteriocins of Proteus vulgaris which were selected at random and tested against 44 P. mirabilis strains. The bacteriocins inhibit from 4 to all 44 P. mirabilis strains.

by Pseudomonas pyocyanea strain P 10. Mukai (1960) found that Escherichia coli 15 produces colicin 15 only when irradiated. Similar results were found for the pyocin produced by P. aeruginosa strain R (Kageyama & Egami, 1962; Kageyama, 1964).

Since ultraviolet irradiation induces lysogeny and bacteriocinogeny, Ben-Gurion (1965) suggested that the primary event that triggers induction may be analogous in both systems, especially after ultraviolet irradiation, since both inductions are photoreactivable. Ben-Gurion (1965) suggested though that the steps which follow the primary event differ. In lysogenic bacteria the immunity breakage is a prerequisite of induction but in bacteriocinogenic bacteria, immunity represents resistance to bacteriocin molecules, which probably act from outside. There is no necessity for immunity breakage before bacteriocin production can proceed. Ben-Gurion (1965) suggests that the induction of bacteriocins is a consequence of cell death due to unbalanced growth of the bacteria as a result of irradiation.

Sixty-seven of the 118 (57%) Proteus vulgaris strains were found to be bacteriocinogenic. This result is similar to that of Cradock-Watson (1965) who found that 61% of 229 P. mirabilis strains produce bacteriocins. Twenty-five per cent of the Escherichia coli strains were found to be colicinogenic (Reeves 1965).

Hamon (1964) found that bacteriocinogeny is strikingly prevalent among bacterial strains. It was found that 30% of the strains of Hafnia species produce bacteriocins active on Hafnia. Seventy-eight per cent of Erwinia species produce bacteriocins when tested against Erwinia indicator strains. In Paracolobactrum arizonae, 15% was noted to produce arizonacins. Of the Enterobacter cloacae strains examined, 27% produce bacteriocins. Eighty-seven per cent of strains of Serratia species produce bacteriocins. Production of bacteriocin in Klebsiella species was found in 34% of the strains. Seventy-five per cent of Aerobacter aerogenes were bacteriocinogenic. Fluocins were found in 43% of strains of Pseudomonas fluorescens.

Ben-Gurion & Hertman (1958) showed that 96% of Pasteurella pestis strains produce bacteriocins.

Hamon, Veron & Peron (1961) showed that a large proportion (96%) of Pseudomonas pyocyanea strains produce bacteriocins and 49% of strains of Listeria monocytogenes was found to produce monocins (Hamon & Peron, 1962).

A wide variety of gram-positive and gram-negative organisms have been studied for the production of bacteriocins and the incidence of bacteriocinogeny is found to vary greatly in the different organisms. It may be that many more organisms produce bacteriocins but as a result of a lack of appropriate indicator organisms, this variation in

the / .....

the incidence of production of bacteriocins is found.

The production of bacteriocins appears thus to be a widespread phenomenon, with the main activity of each family limited to the group of species which produce it. Hamon & Peron (1963) used the spectrum of activity of bacteriocins as an aid in bacterial classification. They argued that as bacteriocins usually act only on strains closely related to the producing strain, the production of bacteriocins by one species which acts on another, indicates a close relationship between these two species. Coetzee (1963) found that many phages isolated on Proteus mirabilis strains also lyse P. vulgaris strains and vice versa. Studies on the deaminases of P. mirabilis and P. vulgaris (Smit & Coetzee, 1967) suggest a close relationship between these two groups. The finding here that the bacteriocins of P. vulgaris only act on strains of P. mirabilis and no other species, is one of the facts which favour a close relationship between these two groups.

Bacteriocinogeny has a certain selective advantage for the bacteria which produce it (Reeves, 1965). In most environments dispersal of a clone shows a strong tendency to be localised. The production of bacteriocin by a few clones will kill the nearby bacteria, which may,for example,be in competition with the bacteriocinogenic clone for nutrients. The prospects of the clone are improved and give the bacteriocinogenic property a selective advantage.

Bacteriocinogenic / .....

Bacteriocinogenic factors also confer immunity on their hosts to the same bacteriocin produced by other clones. Any bacteria which carry chromosomal genes which determine bacteriocinogeny, would also be selected in this way.

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CHAPTER III

PURIFICATION OF *PROTEUS VULGARIS* BACTERIOCINS

CHAPTER III

PURIFICATION OF PROTEUS VULGARIS BACTERIOCINS

INTRODUCTION

Not all bacteriocins which produce good zones of inhibition when grown on agar and cross-streaked, or overlayed with a sensitive strain, produce bacteriocins in broth (Reeves, 1965). Such bacteriocins cannot be obtained in sufficient quantity for characterization and analysis of the properties of the bacteriocins. This liability is found in a few bacteriocins. Marè & Coetzee (1964) described a bacteriocin produced by Alcaligenes faecalis which could be obtained in low titres only by freezing and thawing the agar on which the bacteriocin was produced.

Lachowicz (1965) noted that no staphylococcins were produced in fluid medium. Staphylococcins could only be obtained by the freezing and thawing technique.

The production of bacteriocin in fluid medium was first described by Fredericq (1950). The factors which influence the production of bacteriocins in broth were studied by Matushito, Fox & Goebel (1960) and

Papavassiliou / .....

Papavassiliou (1963). They concluded that the production of bacteriocin in a liquid medium is dependent not only on the organism used, but also on the medium in which the organisms which produce the bacteriocin, is grown.

A method for assay of bacteriocins was first described by Jacob, Siminovitch & Wollman (1952). When they mixed sensitive bacteria with a suitable excess of colicin ML, and titrated samples at intervals, they found that the number of cells which survive decreases exponentially with time of sampling. When the colicin is not in excess the survival curve reaches a plateau that measures the final number of bacteria killed. This proves to be proportional to the amount of colicin added. In this way they measured the number of "lethal particles" in the sample of colicin, and the results are expressed as lethal particles per unit volume of colicin preparation.

Fredericq (1954) used the same principle as Jacob, Siminovitch & Wollman (1952) for the assay of colicins but he spotted drops of a series of successive dilutions of the colicin on the surface of a plate seeded with an indicator strain. In this way he obtained a series of decreasing zones of inhibition ranging from complete inhibition through more and more partial inhibition to normal growth, forming a regular gradient over a range of dilutions. He defined

an arbitrary / .....

an arbitrary unit of bacteriocin activity as the highest dilution of the bacteriocin which still gives a clear zone of inhibition under these conditions.

Hofmeister in 1888 (Green & Hughes, 1962) was the first to use ammonium sulphate for the precipitation of proteins; this led Goebels, Barry, Jesaitis & Miller (1955) to apply a modification of this method for the concentration of colicins. They were able to concentrate and purify colicin K by repeated precipitations with ethanol and ammonium sulphate. Another method of concentration and purification of high molecular weight particles is that of differential centrifugation. MacCullum and Oppenheimer in 1922 (Beard, 1948) were the first workers to use differential centrifugation for the purification and concentration of viruses.

An attempt was made in this study to demonstrate the production of bacteriocins by Proteus vulgaris in fluid medium.

METHODS

Production

Bacteriocinogenic cultures were grown overnight in 50 ml. brain-heart infusion broth, then diluted with 100 ml. brain-heart infusion broth and incubated for 7 hr. After the incubation period

the cultures / .....

the cultures received a small amount of chloroform, were shaken vigorously, centrifuged at 6037g for 30 min. and the supernatant fluids assayed. Samples were also collected at 30 min. intervals and the optical density at 600 m/u determined.

Ultraviolet light irradiation

After dilution of the 50 ml. overnight cultures with 100 ml. brain-heart infusion broth, as described above, the cultures were incubated for 10 min. Ten ml. volumes were pipetted into sterile Petri dishes and irradiated for 4 min. while being gently shaken. The cultures were poured into sterile flasks and incubated in the dark, to prevent photoreactivation (Kelner, 1949; Newcombe, 1955), for 7 hr. These cultures were thereafter treated in the same way as described above.

Assay

Activity in a fluid medium was assayed on SS-agar by a spotting technique (Coetzee, 1967). Serial dilutions of the bacteriocins to be assayed were made in brain-heart infusion broth. A 1/10 dilution of an overnight culture of the indicator organism was made in a sterile Petri dish. Filter paper discs, which had been sterilised in chloroform, were soaked in the indicator organisms and applied to the SS-agar plates for 5 min. After removal of the discs the dilutions

were spotted / .....

were spotted on the plates. The highest inhibitory dilution expressed the titre. (Plate II).

Concentration.

This was effected by salting out the precipitation. The liquid medium containing the bacteriocin was placed on an electromagnetic stirrer while finely powdered ammonium sulphate was slowly added up to 40% (w/v). The solution was left to stand for 2 hr. before centrifugation for 30 min. at 6037g. The pellet was resuspended in 0.1 N-ammonium acetate (pH 7.2) and assayed.

Purification

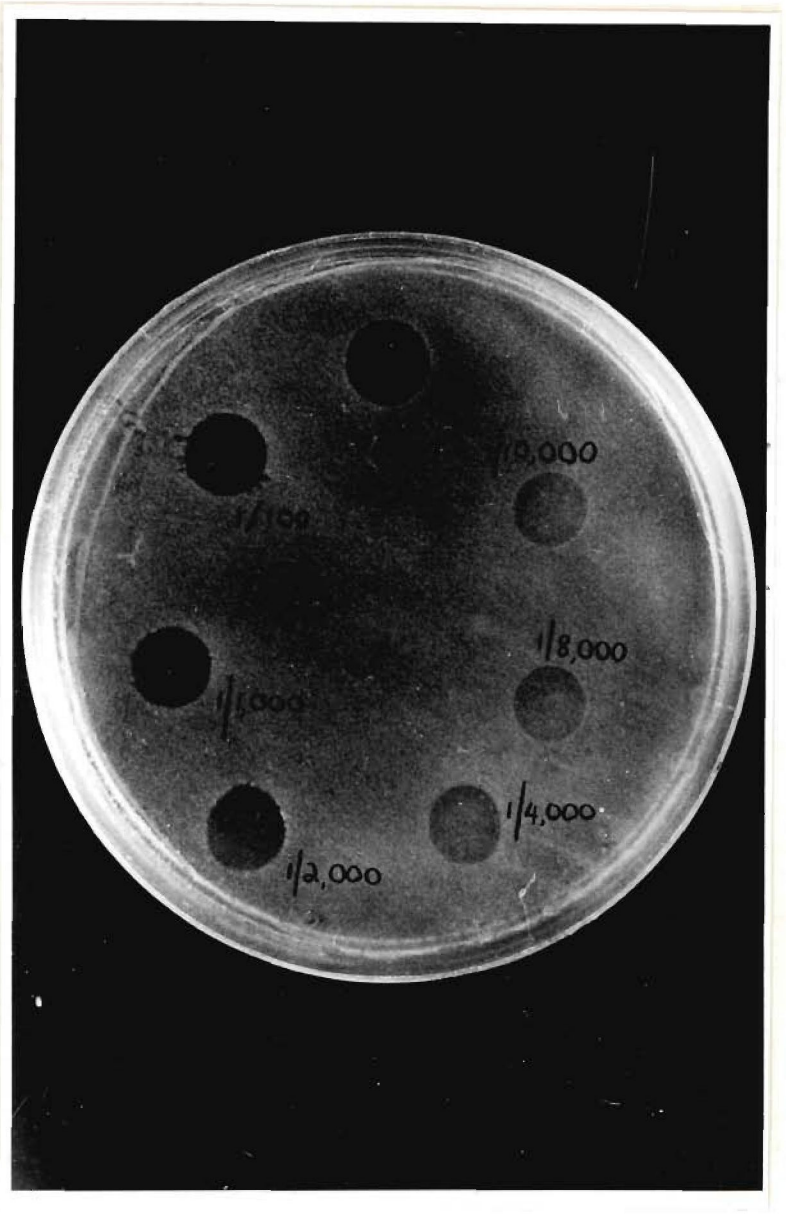
Purification was achieved by several cycles of differential centrifugation. The crude suspension of bacteriocin was centrifuged at 6037g for 30 min. The pellet was discarded and the supernatant fluid centrifuged at 54333g for 2 hr. The resulting pellet was resuspended in 0.1 N-ammonium sulphate (pH 7.2). This procedure was carried out for 2 more cycles and then the bacteriocin activity was finally assayed.

RESULTS

Thirty bacteriocinogenic strains of Proteus vulgaris were

chosen at / .....

PLATE II



Assay of bacteriocin 35. The highest dilution of the bacteriocin which still gives a clear zone of inhibition in the confluent growth of the indicator organism expresses the titre.

chosen at random and tested for inducibility of bacteriocins in fluid medium (Table IV). All these strains produced low titres of bacteriocin in fluid medium but yielded higher titres with ultraviolet induction. All the cultures were subsequently irradiated with ultraviolet light for the production of bacteriocins in fluid medium.

Bacteriocin production was associated with some cell lysis (Fig. 1).

The thirty bacteriocins could be precipitated with 40% (w/v) ammonium sulphate. By means of precipitation the bacteriocins could be concentrated to yield inhibitory titres of about 1/1000.

It was found that the bacteriocins could also be sedimented out of the liquid medium by high speed centrifugation at 54,000g for 2 hr. There was no activity in the supernatant fluids after high speed centrifugation. Hundredfold concentration could be achieved by this method. This result suggests that these bacteriocins are large molecules.

DISCUSSION

The bacteriocins studied here appear to be proteinaceous as they are precipitable with ammonium sulphate. Similar results were found by Goebel & Barry (1958) for colicin K. They were

able to / .....

TABLE IV

Bacteriocinogenic strain No.	Production in fluid medium	
	Without U.V. induction (titre)	With U.V. induction (titre)
11	1/4	1/128
35	1/2	1/128
41	1/4	1/128
49	1/2	1/128
50	1/2	1/128
70	1/4	1/128
71	O	1/128
116	O	1/128
28	O	1/4
36	1/2	1/64
80	O	1/128
105	O	1/4
46	O	1/8
55	O	1/4
52	1/2	1/128
43	1/2	1/16
16	1/4	1/8
4	O	1/4
37	1/2	1/128
60	O	1/4
48	1/2	1/8
47	1/4	1/128
45	1/2	1/128

Continued / .....

Continued / .....

Bacteriocinogenic strain No.	Production in fluid medium	
	Without U.V. induction (titre)	With U.V. induction (titre)
72	1/2	1/8
67	1/4	1/16
75	1/4	1/128
62	O	1/4
61	O	1/4
64	1/2	1/128
68	1/4	1/16

Table IV. Bacteriocinogenic strains of Proteus vulgaris tested for inducibility of bacteriocin production by ultraviolet irradiation.

FIGURE 1

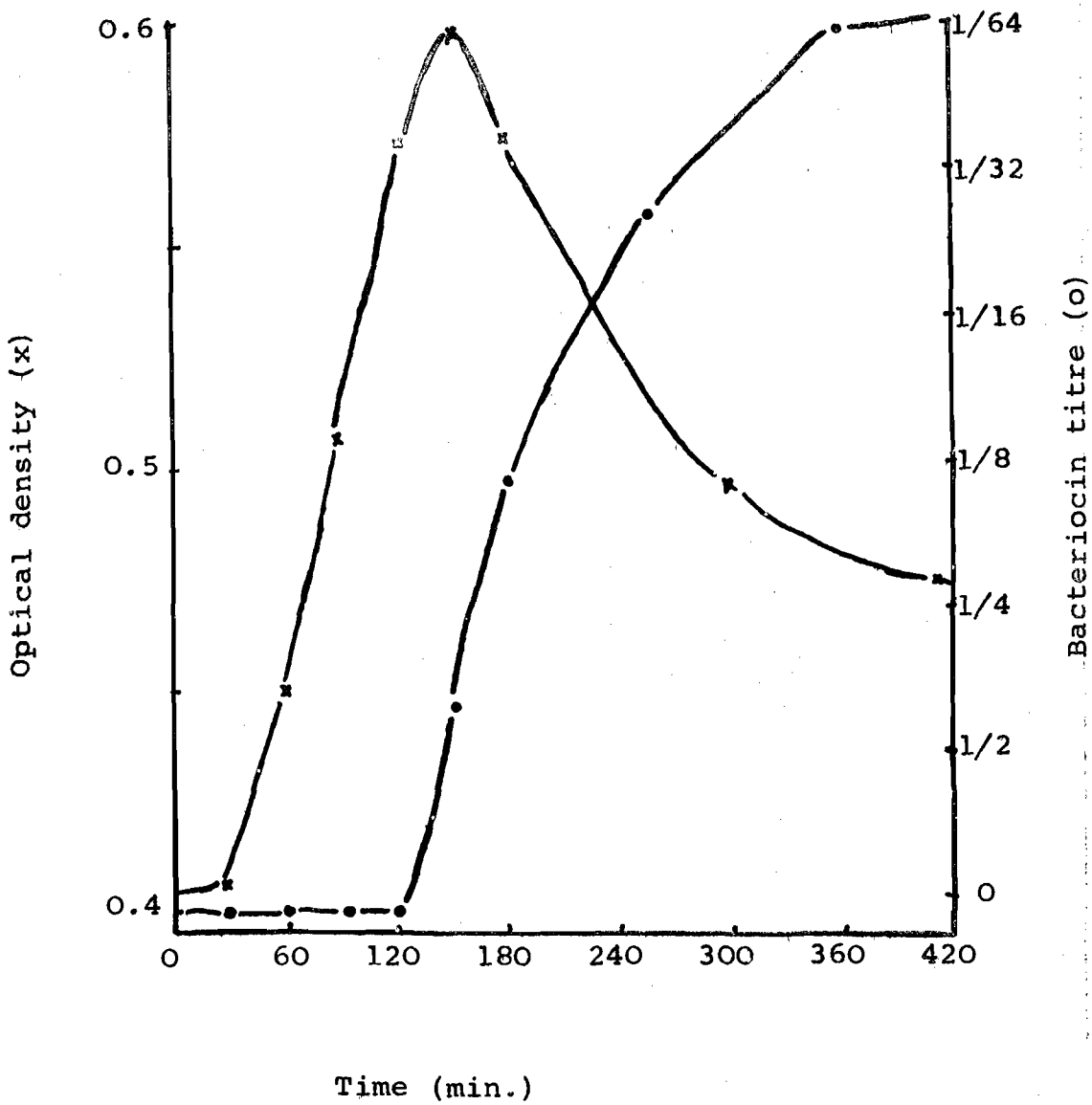


Fig. 1 Time course of bacteriocin production.
Optical density of the induced culture: x - x.
Bacteriocin activity: o - o

able to precipitate and concentrate colicin K with ammonium sulphate.

Colicin A (Barry, Everhart & Graham, 1963), colicin F(E_2) (Reeves, 1963), colicin V (Hutton & Goebel, 1962) and colicin I (Keene, 1966) were all found to be precipitable with ammonium sulphate.

The different pyocins described by Jacob (1954) and Kageyama & Egami (1962) were also found to be precipitable with ammonium sulphate.

The fact that these bacteriocins are sedimentable by high speed centrifugation, places them in the class of large structures. This finding is similar to that found by Kellenberger & Séchaud (1957) for the defective phages of T_2 produced by Escherichia coli strain B. It was found that a concentrated suspension of rod-shaped particles could be obtained from the lysate by centrifugation at 25,000g.

According to Jacob et al. (1953) the definition of bacteriocinogeny is its lethal biosynthesis. The production of bacteriocin involves the death of the bacterium without lysis (Ozeki, Stocker & Margerie, 1959). The finding here that production of bacteriocin is concomitant with cell lysis, is similar to the results obtained by Endo et al. (1965) for colicin 15 and for the pyocin produced by Pseu-

domonas aeruginosa strain R (Kageyama, Ikeda & Egami, 1964). Kageyama, Ikeda & Egami (1964) suggest that a lytic enzyme is synthesized which acts at the time of lysis - the incorporation of the lytic enzyme into the pyocin structure is analogous to that of the bacteriophage (Adams, 1959). These workers were able to demonstrate that a muramidase-like enzyme was synthesized and suggested from immunoligical studies that some of the enzyme may be associated with the pyocin.

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CHAPTER IV

ELECTRON MICROSCOPY OF PROTEUS VULGARIS BACTERIOCINS

CHAPTER IV

ELECTRON MICROSCOPY OF PROTEUS VULGARIS BACTERIOCINS

INTRODUCTION

The electron microscope was developed sufficiently in 1934 to allow Marton (1934) to use it in the photography of bacteria, but it was not until five years later in 1939 that Kausche, Pfankuch & Ruska (Williams & Wyckoff, 1945) were able to demonstrate the appearance of the particles of tobacco mosaic virus. Although the pictures they obtained were indistinct the particles of tobacco mosaic virus were found to be small, slender and rod-like. The support film used by these workers was collodion (nitrocellulose). Schaefer & Harker (1942) described the use of another substance which is stronger and more temperature-stable than collodion and which can support large particles with more success. This substance is known as Formvar (polyvinyl formal). The electron microscopy of viruses received a new stimulus when Williams & Wyckoff (1945) demonstrated the applicability of a newly developed "shadowing" technique. The problem at that time was to increase the contrast between different components of a preparation and between the preparation

and the / .....

and the substrate which supports it. These workers found that "shadow-casting" with a thin film of metal was very effective in revealing the shape as well as the presence of many minute biological objects. The principle was that high points on the surface intercept more than an average number of condensing atoms and at the same time shield areas immediately behind them. These shielded areas, devoid of deposited metal, appear as "shadows" of the elevated regions responsible for them. The metal first used was gold but this was later replaced by a number of other metals such as chromium, copper and nickel.

Another problem which arose with the use of this new technique was to preserve the three dimensional structure of the specimens for electron microscopical examination as surface tension forces were apt to distort the specimens. Williams (1953) developed the technique of "freeze-drying" specimens for electron microscopy. This method minimized the preparative distortions.

The method of "shadow-casting" was used for many years until Brenner & Horne (1959) described another technique for high resolution electron microscopy of specimens. This method consisted of "embedding" the particles in an electron dense material such as potassium phosphotungstate which introduces contrast by negative staining. Carbon-coated grids are used and the method for the

evaporation / .....

evaporation of carbon was developed by Bradley (1954) and is now in general use.

Brenner & Horne (1959) sprayed a mixture of suspension of the specimen and phosphotungstate onto the carbon-coated grids and Bradley & Kay (1960); Bradley (1962) simplified this procedure by their "spreading" technique. Their method is to allow a thin film of liquid to spread over the carbon-film and which is then left to dry on the grid.

As a result of the finding that the bacteriocins described in this study are sedimentable and thus possibly large molecules, it was decided to investigate these bacteriocins under the electron microscope. The effect of oxidation on bacteriocin activity was also investigated. Levinthal & Fisher (1953) observed that ghosts which had been sheared off the bacterial cell wall after absorption, had tails which were shortened by half their original lengths and Lanni & Lanni (1953) mentioned briefly that phage tails may be similarly shortened by oxidation. Kellenberger & Arber (1955) found that as a result of oxidation by treatment with hydrogen-peroxide and alcohol, phage T_2 and T_4 tails could be made to contract and that such a contraction exposed an inner core.

METHODS

Electron microscopy

Bacteriocin samples were suspended in 0.1 N-ammonium acetate (pH 7.2) and negatively stained with a 2% neutral potassium

phosphotungstate / .....

phosphotungstate acid (pH 7,4) (Brenner & Horne, 1959). These suspensions were mounted on carbon support films by a spreading technique (Bradley, 1962). The Formvar film is cast on glass according to the method described by Drummond in 1950 (Bradley, 1965) as follows :-

(a) A standard microscope slide is cleaned with detergent solution and polished with a soft cloth without rinsing the detergent away in water.

(b) The slide is dipped into a 0,3% (w/v) solution of Formvar in ethylene dichloride and drained at a steep angle under cover until dry.

(c) The Formvar film is floated on to a water surface by lowering the slide slowly into a dish of distilled water at a shallow angle. The film parts from the glass by surface tension and floats on the surface of the water.

(d) The film is picked up on grids by holding these in forceps, immersing them in the bath and bringing them up below the pieces of floating film.

(e) After drying on filter paper, each grid is laid on a glass slide and placed in an evaporating unit.

(f) A carbon coating 20 to 100 Å in thickness is now placed on the film, lying on the grid.

(g) The Formvar film substrate is washed away by immersion

in a / .....

in a bath of chloroform.

The preparation of negative-staining mounts was done as follows :- Equal volumes of bacteriocin suspension and negative-staining solution totalling 0.02 ml. were mixed on a glass slide by pipette. A freshly de-greased carbon support film was then touched on to the surface of the mixture. Excess liquid was removed with filter paper until only a thin film covered the grid. After drying, the specimen was ready for examination in a Philips EM 200 electron microscope.

Contraction of phage tail-like structures

Purified bacteriocin was treated with 3% (v/v) H_2O_2 and 10% (v/v) ethanol (Kellenberger & Arber, 1955). Samples were taken at one minute intervals, diluted with 0.1 N-ammonium acetate to stop the reaction, assayed and mounted for electron microscopy. Untreated bacteriocins were used as controls.

Adsorption of phage tail-like structures

Indicator organisms were sedimented from 10 ml. overnight broth cultures and resuspended in 0.5 ml. of purified bacteriocin and in 0.5 ml. bacteriocin treated with H_2O_2 and ethanol. The mixtures were incubated for 10 min., centrifuged at 6037g for 30 min.

and the / .....

and the supernatant fluids assayed and examined in the electron microscope. Sedimented indicator strains were also mounted for electron microscopy. Controls were run concurrently.

Dienes phenomenon

The 118 Proteus vulgaris strains were tested against each other for the Dienes demarcation line between their swarms (Dienes, 1946) as follows :- two of the P. vulgaris cultures were stabbed at opposite ends of a nutrient agar plate and incubated at 25° (Plate III).

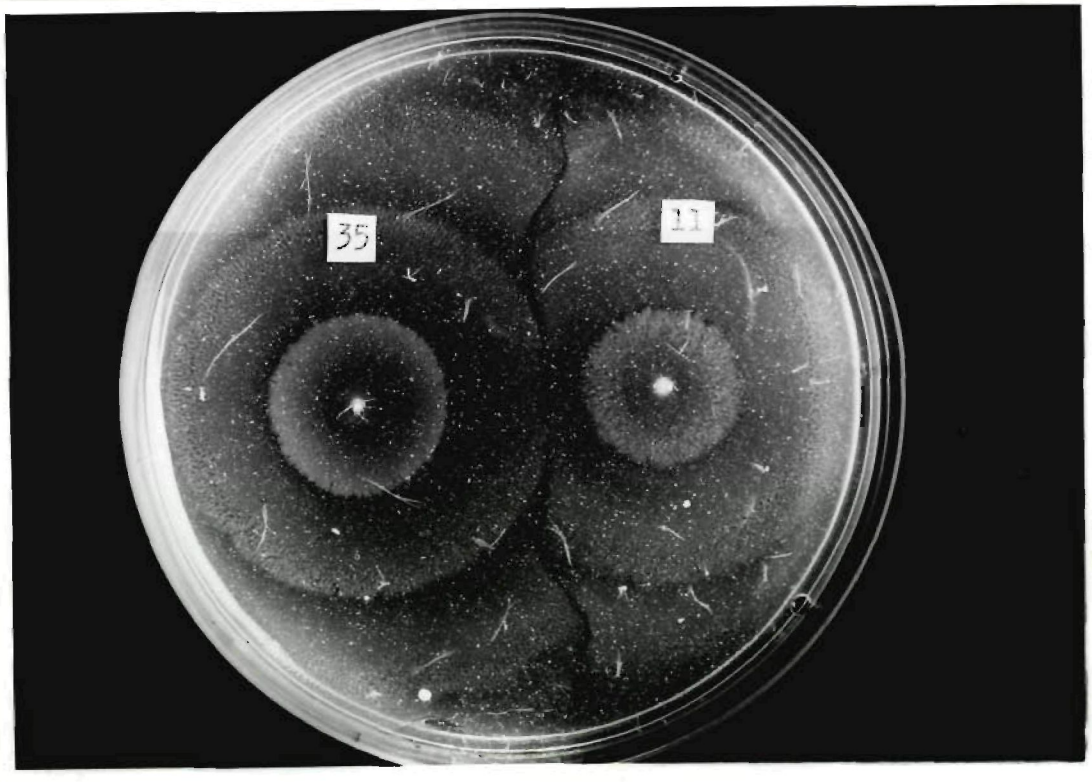
RESULTS

Electron microscopy

Electron microscopy of purified bacteriocins revealed masses of structures resembling sheathed phage tails (Plate IV). Some bacterial debris was usually present. In two of the preparations occasional phage-like particles were also seen. Those in bacteriocin 35 (Plate V) closely resemble Proteus mirabilis phage 13 vir. while the phage-like particles in bacteriocin 71 (Plate VI) look like P. rettgeri phage 7476/332 (Prozesky, de Klerk & Coetzee, 1965). No isolated phage head-like objects were detected. Most of the

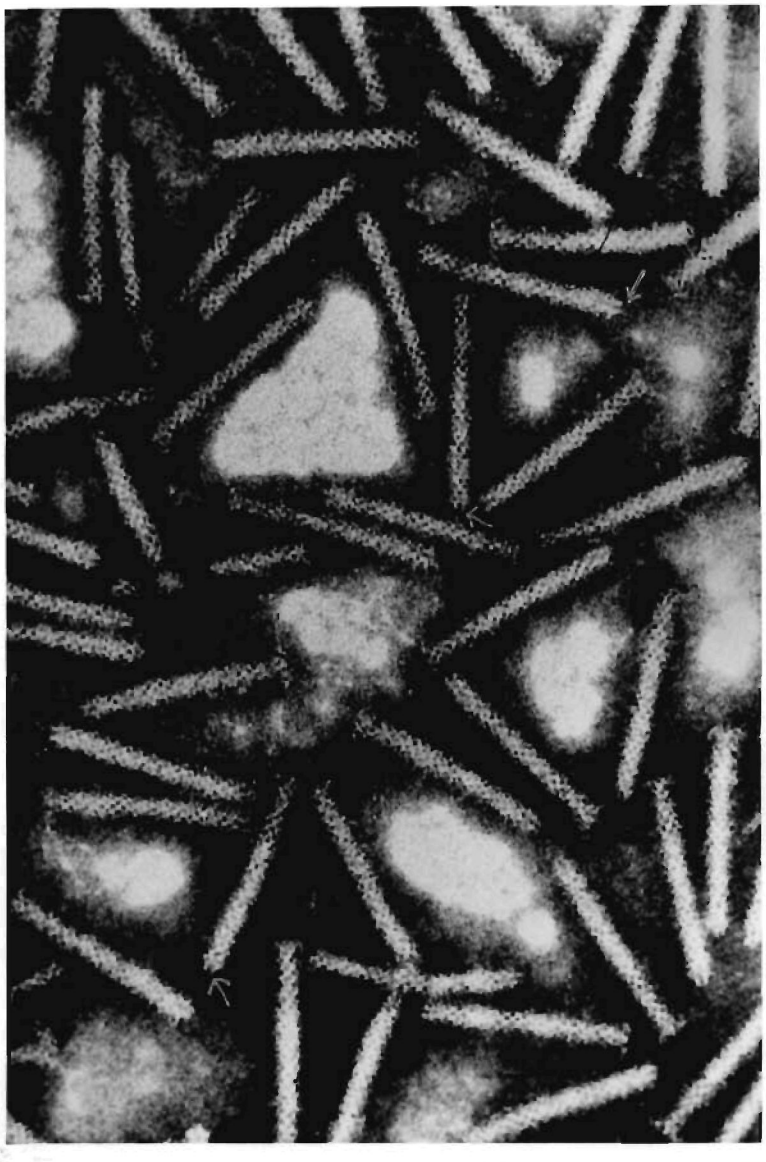
tail-like / .....

PLATE 111



Nutrient agar plate inoculated at opposite ends with Proteus vulgaris strains 35 and 11. The sharp line of demarcation between the swarms denotes a positive Dienes phenomenon.

PLATE IV

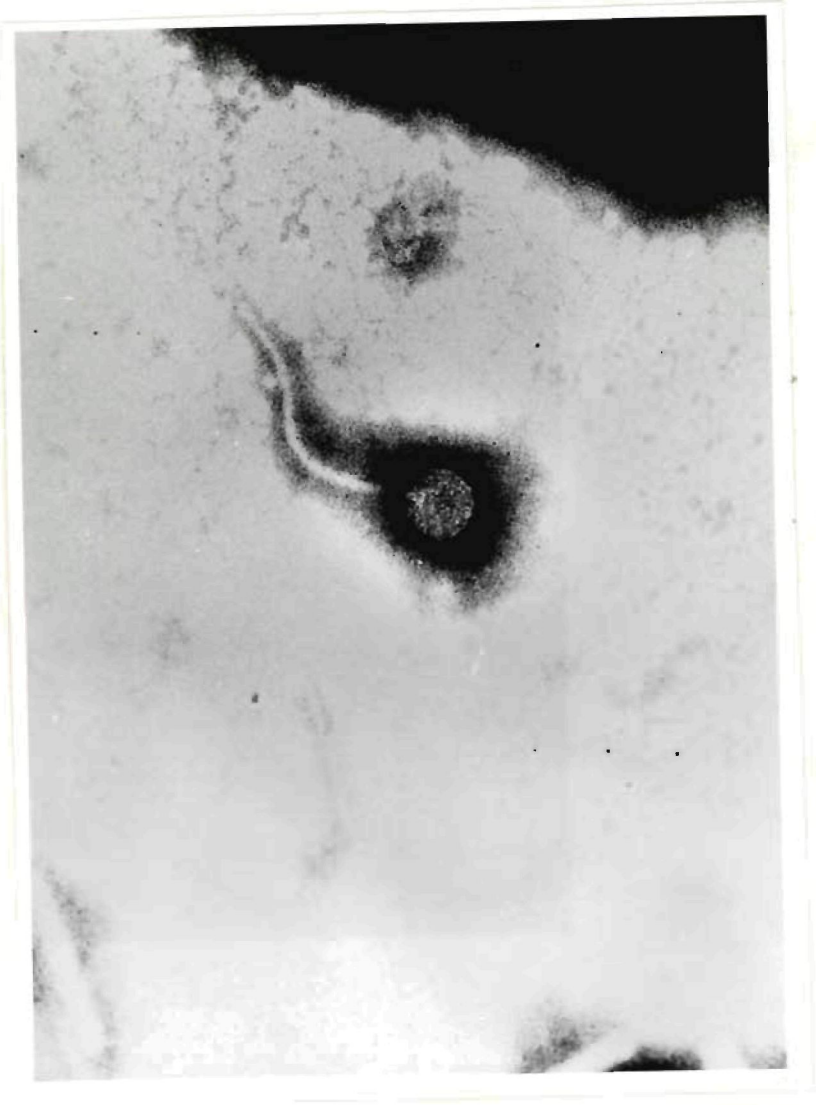


Phage tail-like particles of bacteriocin 45. Arrows indicate projecting cores. This projection appears hollow.

PLATE V

Phage tail-like structures of bacteriocin 35 and a phage-like structure.

PLATE VI



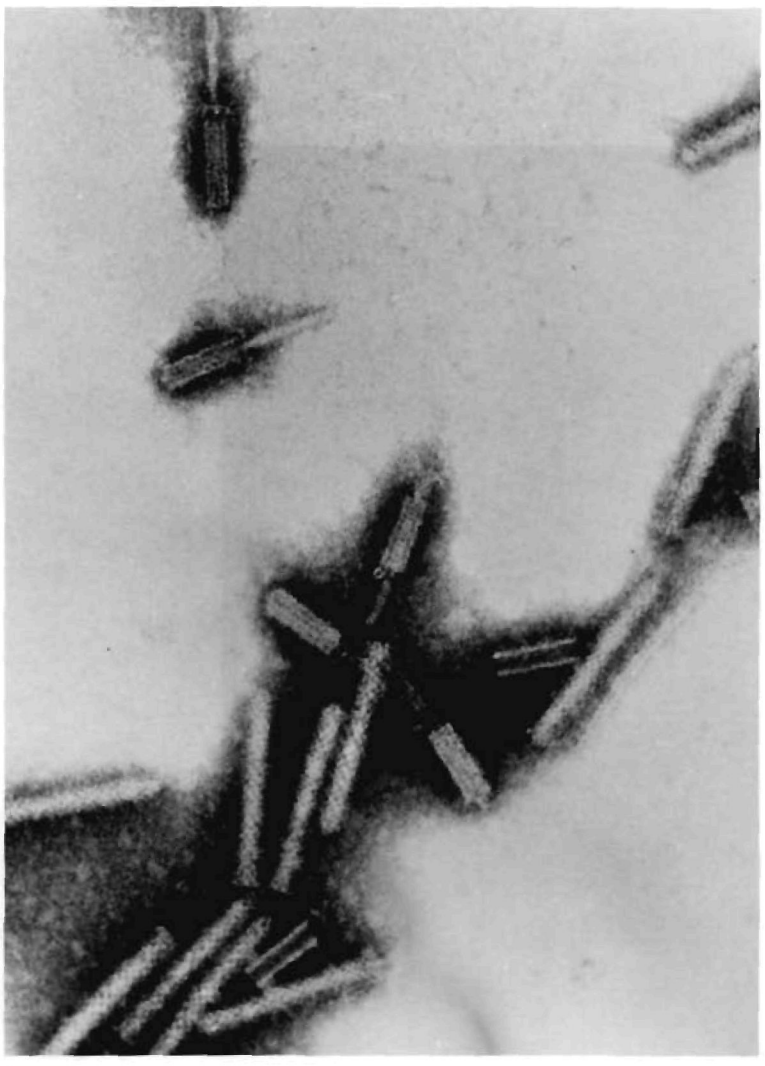
Phage-like structure from bacteriocin 71.

tail-like structures were completely sheathed while in a few the sheaths were contracted and revealed hollow cores (Plate VII). In some cases the core had slipped out of the contracted sheath leaving a short tube (Plate VIII). The hole in this tube is 90 Å in diameter, but the cores themselves are only 70 Å side. As suggested from Plate VIII there is a slight gap between the core and the tube wall of the sheath.

Treatment of the sheaths with H_2O_2 and ethanol caused them to contract and the titre of such preparations was zero (Plate IX). No tail fibres, base plates or tail pins were seen. At one of the ends of the fully-sheathed structures the tail core was seen projecting (Plate IV). This projection appeared hollow (Plate X). The average of 6 to 10 measurements of the tail-like structures of all 30 bacteriocins are identical, 1280 Å in length and 180 Å in width. Contracted sheaths were hollow cylinders 560 Å x 200 Å. Diagonal cross-striations suggestive of helical symmetry could be seen for short distances on some of the extended sheaths (Plate X). In only five preparations, i.e. Nos. 55, 60, 61, 62 and 105 did the direct relationship between the number of structures and titre not hold. These preparations contain numerous fully-sheathed tails but biological activity is low. Five (Nos. 12, 20, 26, 58, 63) of the 48 P. vulgaris strains which did not show bacteriocin activity were also processed for bacteriocin production in parallel with

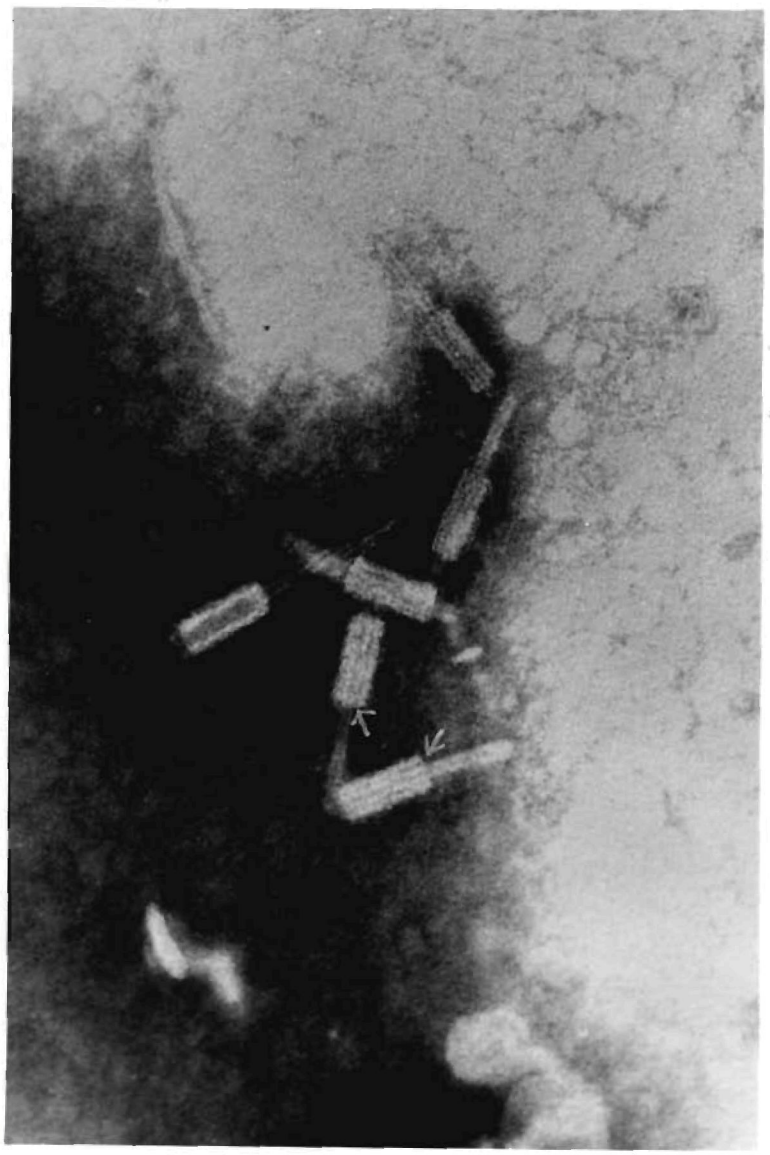
bacteriocinogenic / .....

PLATE VII



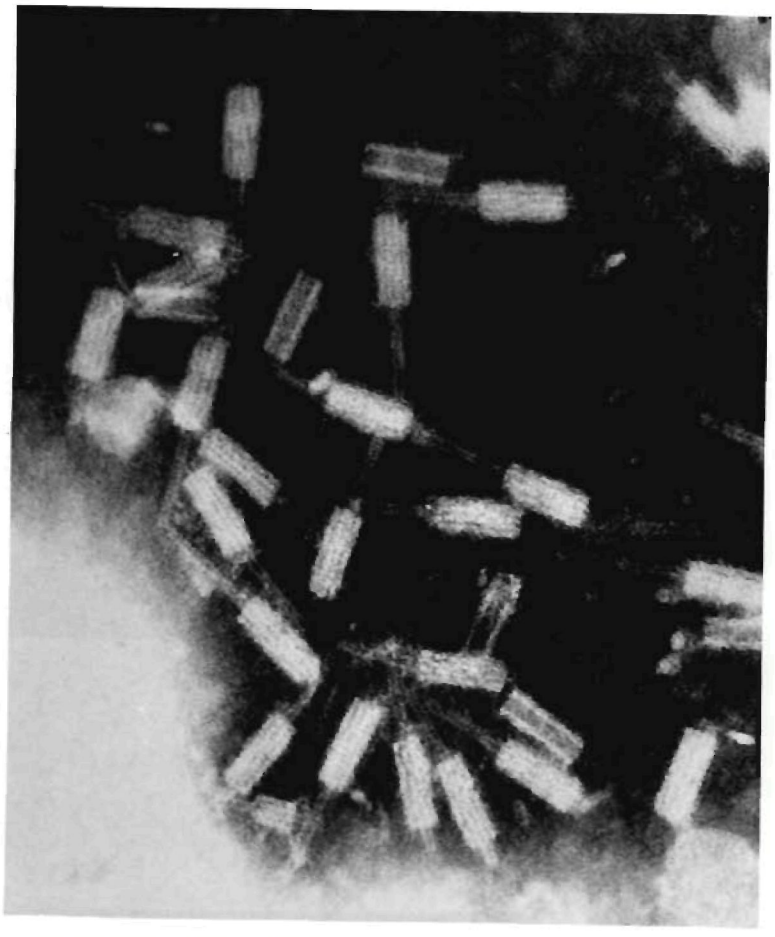
Uncontracted and contracted phage tail-like particles of bacteriocin 45. Contracted phage tail-like particles reveal hollow cores.

PLATE VIII



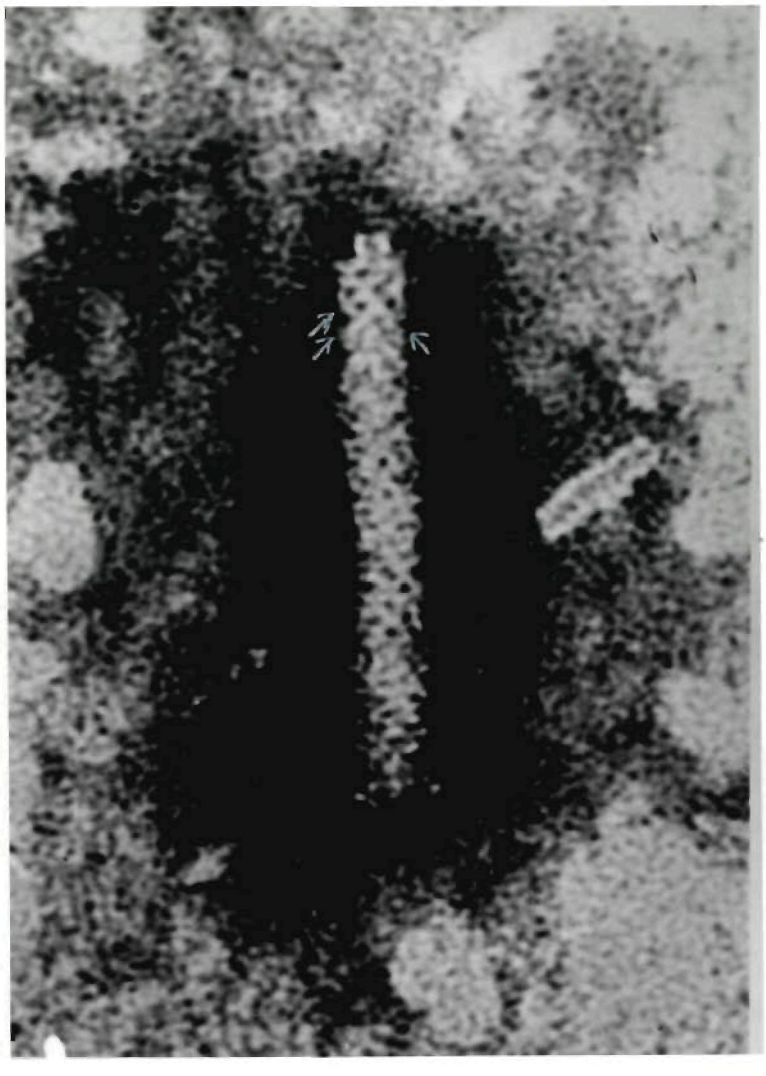
Contracted phage tail-like particles from bacteriocin 11. Arrows indicate slight gap between core and tube wall.

PLATE IX



Phage tail-like structures of bacteriocin 11 treated with H_2O_2 and ethanol. This shows contracted sheaths round hollow cores and empty sheaths.

PLATE X



Phage tail-like structure of bacteriocin 35 with uncontracted sheath and projecting tail core. This projecting appears hollow. Arrows indicate diagonal cross-striations.

bacteriocinogenic strains and examined in the electron microscope. These five preparations showed no phage-like structures.

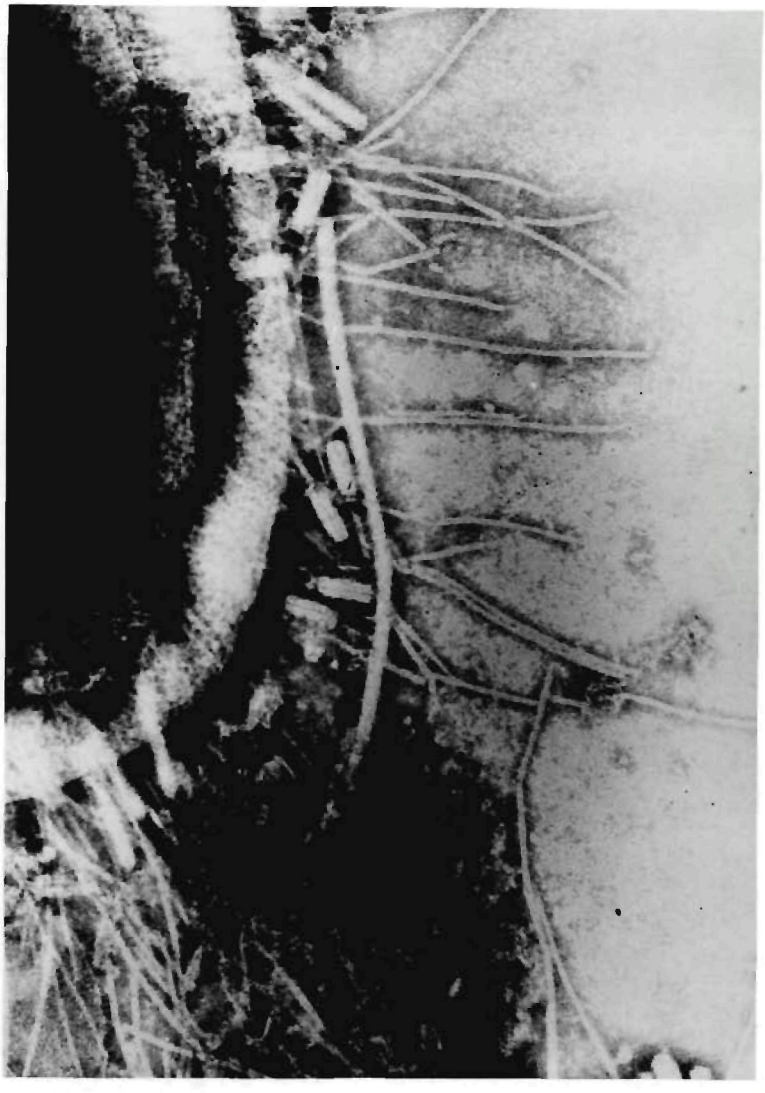
Adsorption of bacteriocins

Suspensions of indicator organisms added to high titre solutions of bacteriocins adsorbed all the activity. This was a specific reaction because suspensions of non-susceptible organisms did not reduce titres of bacteriocins. Electron micrographs of these indicator organisms revealed the tail-like structures around the organisms (Plate XI). The structures tended to be vertically orientated and many were triggered with the core pointing towards the cell. High titre preparations treated with H_2O_2 and ethanol and then adsorbed with suspensions of susceptible organisms, had many contracted tail-like structures in the supernatant fluid and very few around the indicator organisms. This may indicate that triggered tails do not adsorb.

Dienes phenomenon

All the 118 Proteus vulgaris strains differed from one another in the sense that when matched on agar they produced lines of demarcation between their swarms.

PLATE XI



Portion of a cell of Proteus vulgaris strain 10 with bacteriocin 37 around organism. Fully-sheathed and contracted forms are visible between pili. A fragment of a flagellum is also present.

DISCUSSION

Killing activity was always observed to be associated with fully-sheathed forms of the particles during preparation of the tail-like structures. With few exceptions the impression was gained that the number of uncontracted particles bore a direct relation to the activity of the preparations. The five exceptions (Nos. 55, 60, 61, 62 and 105) which contain numerous fully-sheathed tails and in which biological activity is found to be low, may be due to a lack of more susceptible indicator organisms.

The structures described were occasionally associated with phage-like particles which may be temperate phages (Bradley & Dewar, 1966).

These structures also closely resemble the sheathed contractile tail of phage 107/69, a temperate transducing phage of Proteus vulgaris (Coetzee, de Klerk & Smit, 1967).

Taubeneck (1963) considered the phage tail-like structures which are liberated by Proteus mirabilis strain 52 to be the product of defective lysogeny. These particles lack DNA and upon absorption the sheaths contract but the cores do not penetrate the cell wall; they project outwards beyond the sheaths. Shadow-cast preparations were presented and detailed comparison with the structures which are described here is not possible, although comparison with other tail-

like structures / .....

like structures which are described in literature are given in Table V.

TABLE V

Tail-like structures	Measurements of tail-like structures		Reference
	length	width	
Bacteriocin produced by Proteus vulgaris.	1280Å	180Å	–
Colicin 15	1100Å	170Å	Mennigmann (1965).
Pyocin 28	500-4000Å	90Å	Takeya, Minamishima, Amako & Ohnishi (1967
Pyocin produced by Pseudomonas aeruginosa strain R.	1200Å	150Å	Ishii, Nishi & Egami (1965).
Pyocin produced by Ps. aeruginosa strain TTC.	1400Å	180Å	Bradley & Dewar (1966)
Monocin produced by Listeria monocytogenes.	2400Å	200Å	Bradley & Dewar (1966)

Table V: Comparison of the dimensions of Proteus vulgaris tail-like structures with other tail-like structures mentioned in literature.

The fully-sheathed structures of Proteus vulgaris adsorb to bacteria like phage tails. The visible portion of the core of particles with uncontracted sheaths appears hollow. The structures may be empty sheathed phage tails liberated by defective lysogenic P. vulgaris

strains / .....

strains. They possibly kill bacteria in the same way as do the ghosts of T_2 phage. These ghosts puncture the cell wall and a leakage of cell contents results which leads to cell death (Herriot, 1951). Kellenberger & Séchaud (1957) demonstrated that core-like structures of phage T_2 adsorb to but do not kill Escherichia coli B while similar structures derived from T_4 do not adsorb to Escherichia coli B. Arber & Kellenberger (1958) also demonstrated that phage tail-like structures produced by defective lysogenic strains of E. coli K_{12} adsorb to a strain C 60. Triggered P. vulgaris phage tail-like structures like the structures derived from phage T_4, do not adsorb.

The 5 strains which have no killing effect and do not produce phage-like structures still form a Dienes demarcation line (Dienes, 1946) between their swarms on agar. Hughes (1957) suggested that there is some toxic effect when one strain acts on another. He concluded that there are several chemical agents concerned in the swarming of Proteus. The finding here that non-bacteriocinogenic strains still form a Dienes demarcation line, eliminates these structures as a possible cause of the Dienes phenomenon.

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CHAPTER V

PROPERTIES OF PROTEUS VULGARIS BACTERIOCINS

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INTRODUCTION

A method used for the separation and identification of bacteriocins is agar gel electrophoresis. Ludford & Lederer (1953) demonstrated, with the use of a simple apparatus, that colicins consisted of complexes, the components of which could be separated by means of electrophoresis inside agar gel. The possibilities of this method, in typing strains of bacteriocinogenic organisms and for the study of bacteriocins, were realised by Chapple (1962), who described a modified version of the method of Ludford & Lederer (1953). The basic principle of electrophoresis is that a particle is electrically neutral at its isoelectric point. The isoelectric point is the pH at which positive and negative charges of the particle are in an equilibrium. Different particles may differ from one another in their isoelectric points. By changing the pH in which the particle is suspended and thereby changing the charge carried by the particle, it becomes mobile in an electric field. Particles which have migrated and which form a single sharp boundary are interpreted to indicate homo-

geneity / .....

geneity of the material (Beard, 1948).

Gratia & Dath (1926) were the first to use diffusibility as a criteria for identification of bacteriocins. They found that colicin V passes through cellophane membranes but colicin A does not. Fredericq (1948) modified this method. Colicins were placed directly in the agar and allowed to diffuse for a standard length of time and then the diameter of the inhibitory zone measured.

Gratia (1932) found that colicin V is heat resistant but found that the activity of the other groups of colicins are destroyed at a temperature of 70°. He also suggested that the action of proteolytic enzymes could be used as another criteria for the identification of bacteriocins. Gratia & Betz-Bareau (1946) were the first to study the action of trypsin on different colicins. They observed that there was a certain similarity between the response of the different colicins to the action of trypsin.

In 1922 Wu proposed the use of the Folin phenol reagent for the measurement of proteins. Lowry, Rosebrough, Farr & Randall (1951) modified the analytical procedure which utilises this reagent. This method is generally used for protein estimation. The reaction obtained by heating carbohydrates with anthrone in sulphuric acid, was first described as a qualitative test by Dreywood in

1946 (Scott & Melvin, 1953). This method was used by Scott & Melvin (1953) for the determination of concentration of dextran solutions. They also designed a procedure to improve the precision of the test.

Dische in 1930 (Burton, 1956) was the first to use colour reactions for the determination and identification of deoxyribonucleic acid. The reaction consisted of a mixture of diphenylamine and acetic and sulphuric acids at 100°. Burton (1956) modified this method by adding acetaldehyde to the reagents. He found the modified version 3.5 times more sensitive than the original method devised by Dische.

The possibility that heptoses and their esters may be intermediates in various metabolic processes in animal and plant tissues suggested to Dische (1953) that sensitive colour reactions should be developed for the detection and microdetermination of heptoses. He devised a sensitive qualitative and quantitative colorimetric method for the determination of heptoses based on the fact that heptoses with orcinol in dilute sulphuric acid produce two characteristic coloured compounds which differ greatly in their adsorption spectra. This method is generally used for the estimation of ribonucleic acids.

METHODS

Agar electrophoresis

This was done according to the method of Marè, Coetzee & de Klerk (1964). 0.7% Difco MacConkey agar was poured into a glass electrophoresis plate (12" x 12" x $\frac{1}{2}$") to a thickness of 2 mm. After the plate had set the bacteriocins were spotted 1 inch from each other along the centre line of the plate. 5% MacConkey agar was poured into two perspex buffer compartments with inside measurements of 12" x 2" x 3/4". After the MacConkey agar blocks had set, the blocks were removed from the buffer compartments and used as supports between the agar plate and the buffer compartments. The buffer used was Difco MacConkey broth. By means of electrodes immersed in the buffer liquids in the two compartments a potential of 70 volts was applied to the system for 18 hr. using a direct current supply from locally constructed apparatus. The current consumed under these conditions was approximately 130 m amp. The electrophoresis was conducted at 4^{o}. The extent of electrophoretic mobility was measured by applying the appropriate indicator organisms to the plate by means of strips of filter paper which had been soaked in a 1/1 diluted suspension of the indicator organism. The plate was incubated overnight.

Diffusibility / .....

Diffusibility

A few drops of bacteriocin were pipetted into a sealed hole in a MacConkey agar plate and the plate kept at 4° for 18 hr. The extent of diffusion was measured by the application of an indicator organism to the plate (Coetzee, 1967).

Action of trypsin

Two samples of 0.2 ml. of each bacteriocin were added to 0.5 ml. of a 200 mM-sodium phosphate buffer (pH 7.5). While one sample was held as a control, the other received 0.05 mg./ml. crystalline trypsin (British Drug House Ltd.; BDH). Both samples were then incubated for 3 hr., and the titres determined.

Heat sensitivity

One ml. of each bacteriocin solution was pipetted into a sterile glass tube, heated from 25° to 70° in a water bath for 20 min. Samples were taken at temperatures of 40°, 50° and 60° and assayed for activity.

Protein estimation

This was done according to the method of Lowry et al. (1951.

Reagents:- / .....

Reagents:-

A: 2% Na_2CO_3 in 0.10 N-NaOH.

B: 0.5% $CuSO_4 \cdot 5H_2O$ in 1% sodium potassium tartrate.

Mix 50 ml. of Reagent A with 1 ml. of Reagent B. Discarded after one day.

C: alkaline copper solution.

E: diluted Folin reagent.

Procedure

Titrate Folin-Ciocaltue phenol reagent with NaOH to a phenolphthalein end-point. On the basis of this titration dilute the Folin reagent (about 2-fold) to make it 1 N in acid. A solution of crystalline bovine albumin was used as standard (Armour Pharmaceutical Co., Kaukakee, Ill., U.S.A.).

A sample (0.3 ml.) of bacteriocin was mixed with 3 ml. of reagent C and allowed to stand for 10 min. at room temperature. 0.30 ml. of reagent E was added and immediately mixed. After 30 min. the optical density was measured at 750 m/u.

Nucleic acids estimation

RNA estimation

This was done according to the method of Dische (1953).

Reagent: / .....

Reagent: 100 mg, $FeCl_3.6H_2O$ in 100 ml. HCl (specific gravity 1.186). To this is added 3.5 ml. of 6% solution of orcinol in ethanol.

Procedure:

To 1.5 ml. of bacteriocin solution is added 3 ml. of the reagent. The reaction mixture is heated at 100° for 3 min. and cooled in tap water. D(-) ribose (BDH) as standard and a blank was run simultaneously. The optical density was read at 665 mμ.

DNA estimation

The method used by Burton (1956) was followed.

Reagent: To 1.5 g. diphenylamine in 100 ml. glacial acetic acid was added 1.5 ml. concentrated H_2SO_4. Before use, 0.1 ml. of aqueous acetaldehyde (16 mg./ml.) is added for each 20 ml. of reagent required.

Extract: Purified bacteriocin (titre 1/4,000) was extracted twice as follows: To 0.5 ml. bacteriocin was added 0.5 ml. I N-perchloric acid (final concentration 0.5 N) and left for 15 min. at 70°. This was repeated with the same volume of 0.5 N perchloric acid and the centrifuged precipitate was extracted at 70° for another 15 min. One ml. of the combined extract (which was made up to 10 ml. in a stoppered measuring cylinder) was used and 2 ml. of the diphenylamine reagent added incubated for 16 hr. at 30°.

The optical / .....

The optical density was read at 600 mμ and compared to 2-deoxy-D-ribose (BDH) as standard.

Carbohydrate estimation

Total sugars were determined by the anthrone method of Scott & Melvin (1953).

Reagent: 0.2 g. of anthrone was dissolved in 100 ml. of concentrated H_2SO_4. The reagent was freshly prepared.

Procedure:

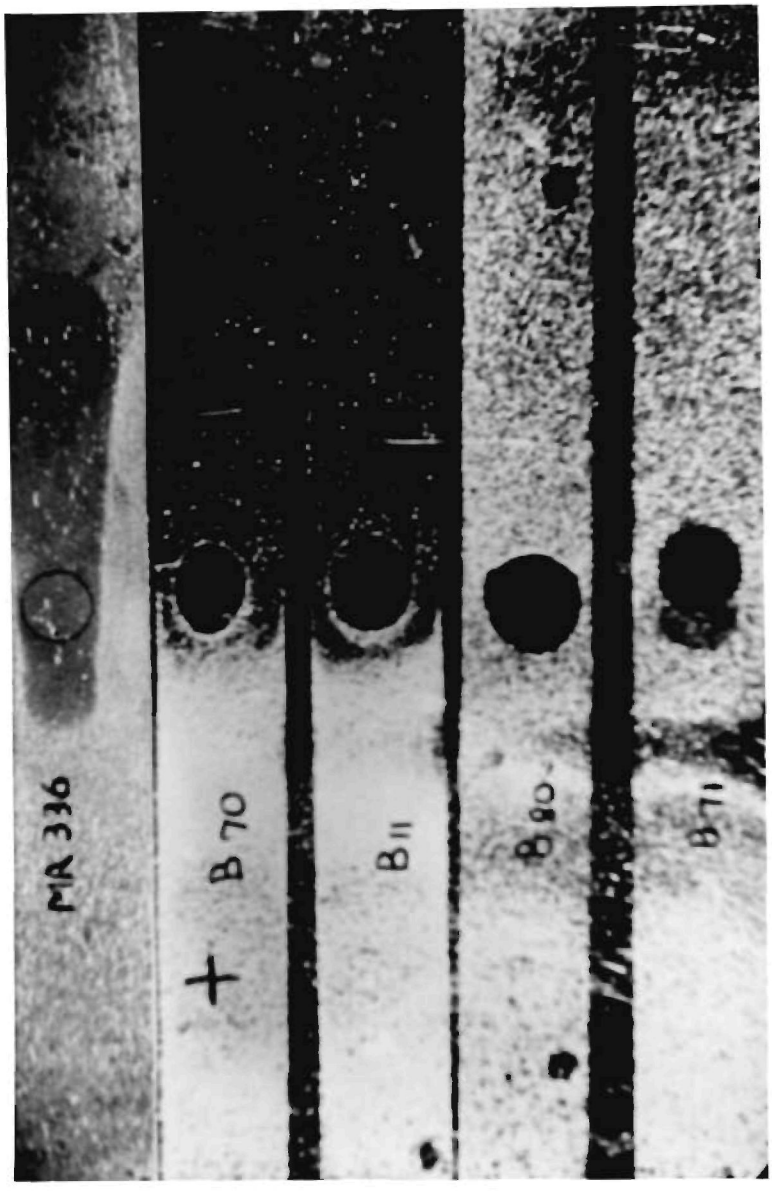
Two ml. of the anthrone reagent was poured into each of two wide-mouthed test tubes and chilled in a water bath at 10°. One ml. of the bacteriocin solution was carefully layered over the H_2SO_4 in one test tube and allowed to chill. The other acted as control. The tubes were shaken vigorously while still immersed in the bath. The samples were brought to room temperature and placed in a bath at 90° for 16 min. They were then cooled and read shortly afterwards at 625 mμ.

RESULTS

The bacteriocins were electrophoretically immobile under conditions where Proteus morganii bacteriocin MR 336 (Coetzee, 1967 and unpublished) moved 6 cm. towards the cathode (Plate

XII). / .....

PLATE XII



Bacteriocins 70, 11, 80 and 71 are immobile in an electric field while a Proteus morganii bacteriocin MR 336 (Coetzee, 1967) shows movement towards the cathode.

XII).

All 30 bacteriocins diffused about $\frac{1}{2}$ cm. from the hole in the agar during 18 hr. Phage 107/69 (Coetzee, de Klerk & Smit, 1967) tested under similar conditions did not diffuse (Plate XIII).

Bacteriocins 50 and 116 were tested for their susceptibility to trypsin. In both cases the titres were reduced 100-fold (Table VI),

TABLE VI

Action of Trypsin

	Bacteriocin 50	Bacteriocin 116
Trypsin	1/10	1/100
Control	1/1,000	1/2,000

Bacteriocins with titres of 1/10,000 were completely inactivated when exposed for 30 min. to a temperature of 60°. (Table VII),

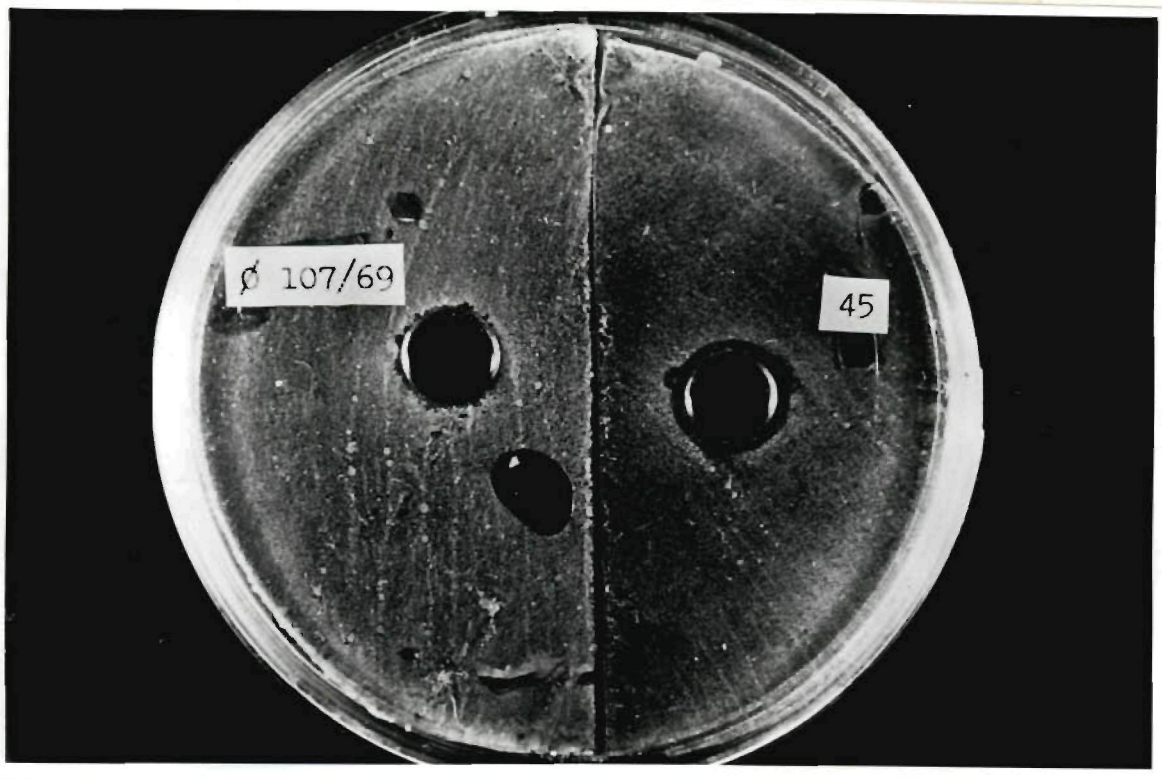
TABLE VII

Heat sensitivity

Temperature (30 min.)	Titre
25°	1/10,000
40°	1/10,000
50°	1/10,000
60°	0

Chemical /

PLATE XIII



Diffusibility of bacteriocin 45. Area of inhibition of growth due to diffusion of bacteriocin, compared with phage 107/69 (Coetzee, de Klerk & Smit, 1967), which does not diffuse through agar.

Chemical analysis of bacteriocin 45, with a titre of 1/4,000 showed it to consist of :-

Protein	640 µg./ml.
DNA-phosphorous	0.0 µg./ml.
RNA-phosphorous	0.48 µg./ml.
Total sugars	412 µg./ml.

DISCUSSION

Bacteriocins range from molecules with sedimentation coefficients of 2.8 S for a Lactobacillus fermenti bacteriocin (de Klerk & Smit, 1967) and 3.6 S for colicin E_2 (Reeves, 1965) through the phage tail-like components of pyocins with an S-value of 90.5 (Kageyama, 1964) to the phage-like colicins 15 and H (Endo et al., 1965; Bradley & Dewar, 1966).

Kageyama (1964) was able to estimate the molecular weight (or particle weight) of the pyocin produced by Pseudomonas aeruginosa strain R. He calculated the molecular weight to be 8,800,000. The molecular weight of the bacteriocin described in this study cannot be calculated as necessary details such as S-value and diffusion constant is lacking. An attempt is being made to obtain this information.

The bacteriocins described here were found to be electro-

phoretically / .....

phoretically immobile. These bacteriocins diffuse through agar and the electrophoretic immobility does not result from an inability to penetrate the agar lattice. On the other hand Kageyama (1964) demonstrated by means of paper electrophoresis that the pyocin produced by Pseudomonas aeruginosa strain R is electrophoretically mobile. The inability to demonstrate mobility in an electric field in this study may be due to the difference in methods applied. Although other bacteriocins have been shown to move on agar with the use of the method described here (Marè & Coetzee, 1964).

The presence of sugars in the chemical analysis of the bacteriocins may be ascribed to bacterial cell walls which usually contaminate preparations of bacteriocins, and the presence of RNA may be due to contamination of bacterial ribosomes (De Ley, 1963).

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CHAPTER VI

GENERAL DISCUSSION

A striking feature which became evident from this work is that all of thirty killing strains of Proteus vulgaris investigated, produce phage tail-like structures. These strains were selected for study merely on the basis of different ranges of activity and it is possible that all sixty-seven of the killer strains produce these tail-like structures. Taubeneck (1963) suggested that the phage tail-like structures which strains of P. mirabilis liberate may be the products of defective lysogeny. If these structures are accepted as the products of defective lysogeny, it is possible that this latter state (Campbell, 1961; Neubauer, 1967) has arisen through selection only of genes which are favourable for the bacterium. These genes can be acquired by transduction (Coetzee, Smit & Prozesky, 1966; Coetzee, de Klerk & Smit, 1967) which permits the formation of recombinant types adapted to a particular local environment and thus increases the selective value of the host species, or by conversion (Coetzee, 1961) which also imparts a selective advantage to the host species. Genes thus acquired will lead to the gradual accumulation of defective prophages in originally lysogenic Proteus.

From the / .....

From the results obtained in this study and those mentioned in literature, it can be said of bacteriocins that two kinds exist: the one kind, phage-like or phage-component-like particles and the other smaller molecules. Bradley & Dewar (1966) suggest that the general concept and classification of bacteriocins may have to be changed. For the present we would advise against naming as bacteriocins newly discovered phage-like or phage-component-like particles with non-transmissible killing activity.

There are a few similarities among bacteriocins, their bacteriocinogenic factors, bacteriophages and fertility factors. These similarities fall into three types (Reeves, 1965). All bacteriocins, bacteriophages and fertility factors probably function through the use of specific "receptors" on the bacterial cell surface. Furthermore, the genetic determinant of bacteriocins, fertility factors and bacteriophages is a small piece of DNA capable of independent replication of the bacterial chromosome, and lastly there is a similarity in the induction behaviour of the inducible bacteriophages and bacteriocins. These similarities suggest some relationship between the various entities mentioned.

Bacteriocinogenic factors behave like temperate phages but their products, the bacteriocins, behave more like obligatory virulent phages (Fredericq, 1963). Bacteriocins could possibly be

virulent / .....

virulent bacteriophages so defective that they can no longer mature into infective particles, but still retain the gene for lethal protein synthesis or, on the other hand, they could have originated through the addition of a bacteriocinogenic factor to a temperate phage genome.

Bacteriocinogenic factors also bear some relationship to fertility factors (Reeves, 1965). The killing property of bacteriocins is usually limited to bacteriocins of the same species and it thus immediately suggests a sex factor gone wrong. In this way bacteriocins could be fertility recognition sites which were modified by natural selection so as to elicit a lethal response from the organism which produces it.

The typing of pathogenic organisms by bacteriocin production and susceptibility of organisms may be a useful tool in the study of epidemiology. Abbott & Shannon (1958) and Gillies (1964) realised this fact when they studied outbreaks of dysentry due to Shigella sonnei. Wahba (1963) studied the production of bacteriocins by strains of Pseudomonas pyocyanea and as a result Darrell & Wahba (1964) were able to develop a typing scheme based on pyocin production. Possibly a similar typing scheme could be developed for the Proteus group. This might be useful in the investigation of infection by Proteus species in burns, wounds and the urinary tract. Cradock-Watson (1965) attempted to type Proteus mirabilis according to bacteriocin production. He was able to

recognise / .....

recognise three types of P. mirabilis and suggests that the discovery of additional indicator organisms may increase the proportion of typable strains and lead to the development of a typing scheme of epidemiological value.

The practicability of phage typing depends on one of the most important properties of phages, i.e. their host specificity. With the discovery of the Vi antigen of Salmonella typhi (Felix & Pitt, 1934), Graigie & Yen (1938) were able to develop the Vi-phage typing scheme for S. typhi. This method is now recognised as the method for epidemiological studies of S. typhosa. It has also established itself as the model for the development of all subsequent schemes for the typing of bacteria by phage. The division of strains of Shigella sonnei by phage typing has been attempted by Hammarström (1947) and Tee (1955) but it was found in practice to be unsatisfactory for epidemiological purposes due to the type instability and large numbers of strains belonging to the same type. Abbott & Shannon (1958) developed a method of typing Sh. sonnei according to the various patterns of inhibition, produced by the bacteriocins of Sh. sonnei on a selected set of sensitive strains. The results were found to be consistent with the epidemiological information they had obtained. In some instances where phage typing cannot be used, bacteriocin typing may be a useful means for the classification of bacteria and this method of typing P. vulgaris will be investigated in the near future.

Bacteriocinogenic / .....

Bacteriocinogenic cells are immune to the killing or biochemical action of homologous "external" bacteriocins. Immunity is not a result of lack of adsorption of bacteriocins onto "receptors" as Maeda & Nomura (1966) demonstrated with radioactive colicins, but is probably the result of the synthesis of a specific immunity substance which interferes with the transmission (Nomura, 1967). In the case of λ-lysogenic cells, both immunity to superinfecting homologous phage and repression of prophage functions are affected by the same substance which interacts directly on the injected DNA (Jacob & Campbell, 1959). Such immunity is a necessity for the existence of lysogeny, since any culture of lysogenic bacteria sensitive to infection and lysis by the temperate phage it carries, would soon be destroyed by a chain reaction of successive bacteriophage growth cycles initiated by the first infective phage particle that appeared in the culture upon spontaneous prophage induction of one of the lysogenic cells (Stent, 1963).

Further investigations arising from this study

From the results that have been obtained in this study a number of interesting points have arisen and it is felt that these justify further investigation.

It is intended to cure the bacteriocinogenic cells of their

bacteriocinogenic / .....

bacteriocinogenic factors and to determine whether there are any antigenic differences between the cured Proteus vulgaris organisms and the corresponding bacteriocinogenic organisms. Uetake, Luria & Burrous (1958) found conversion by phage ϵ^{15} of Salmonella anatum from one antigenic class to another, and that the original surface configuration is once more regained with the loss of the phage. It is also intended to investigate whether the cured strains of Proteus vulgaris still remain hosts to the same P. vulgaris bacteriophages (Coetzee, 1963) as the original bacteriocinogenic strains.

The mapping of various markers by means of transduction will be attempted in future (Coetzee, Smit & Prozesky, 1966). With the use of these genes as markers, the mapping of the bacteriocinogenic factor as well as the transfer of bacteriocinogeny to non-bacteriocinogenic cells by means of the Proteus vulgaris transducing phage 107/69 (Coetzee, de Klerk &Smit, 1967) will also be attempted.

Cradock-Watson (1965) was able to type Proteus mirabilis into three types with the use of production of bacteriocin and susceptibility of organisms. A similar bacteriocin typing scheme for Proteus vulgaris is planned.

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