Bacteriophage-tail-like Particles Associated with Intra-species Killing of *Proteus vulgaris*

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**SUMMARY**

One hundred and eighteen different strains of *Proteus vulgaris* were investigated for bacteriocinogeny. These and an additional 44 strains of *P. vulgaris* were used as indicators. Sixty-seven of the strains had a non-transmissible killing effect on one or more of the indicator organisms and 30 of these 67 bacteriocins with different spectra of activity were further investigated. Individual bacteriocins killed from 5 to 87 of the *P. vulgaris* indicators and a number of 44 different *P. mirabilis* strains but had no action on strains of other species of the family *Enterobacteriaceae*. Broth cultures of bacteriocinogenic strains are inducible by ultraviolet light and yield bacteriocin titres of about 1/100. Activity is sedimentable by high-speed centrifugation. Electron microscopy of all 30 preparations revealed similar phage-tail-like structures with a contractile sheath round a hollow core. The structures consisted of protein and did not contain DNA. The particles resembled some pyocins and also the tail of a *P. vulgaris* transducing phage. In 2 preparations a few phage-like particles resembling other Proteus phages were also seen. Bacteriocin activity was always associated with uncontracted sheaths, and triggered tails did not adsorb to susceptible organisms. We conclude that the tail-like structures are the products of defective lysogeny. The high incidence of the latter state may be accounted for by the selection of genes favourable to the host which were originally acquired through transduction by lysogenization or lysogenic conversion.

**INTRODUCTION**

Bacteriocins are proteinaceous antibiotics with a range of activity usually limited to members of the same species as the producer bacteria and they kill without multiplication (Reeves, 1965). 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. Thus colicin 15 produced by *Escherichia coli* strain WTI5 consisted of small-headed phage-like particles (Endo et al. 1965; Sandoval, Reilly & Tandler, 1965) and the pyocin produced by *Pseudomonas aeruginosa* strain R resembled headless contractile tails of bacteriophages (Ishii, Nishi & Egami, 1965). Bradley & Dewar (1966) ascribed the colicin H activity of *Escherichia coli* A10 to phage-like particles similar to colicin 15. They proved that 3 other pyocinogenic strains of *P. aeruginosa* liberated structures like
those of strain R and that a monocin liberated by a strain of Listeria monocytogenes consisted of phage-like particles. Takeya et al. (1967) showed that pyocin 28 consisted of cross-striated rods about 1000 Å in length. Strain 52 of Proteus mirabilis was found (Taubeneck, 1963) to liberate phage-tail-like structures which were contractile and killed some P. mirabilis and P. vulgaris strains. Van Iterson, Hoeniger & Nijman van Zanten (1967) in an investigation of microtubules in 2 strains of P. mirabilis demonstrated that both strains when induced by mitomycin C produced phage-tail-like structures similar to the pyocins described by Ishii et al. (1965). Cradock-Watson (1965) described bacteriocin production in 139 of 229 strains of P. mirabilis and 1 of 10 strains of P. vulgaris. We decided to test more strains of the latter species and to investigate the nature of bacteriocins discovered.

METHODS

Media. The media were Difco brain-heart infusion broth, MacConkey agar, SS-agar and a nutrient agar.

Bacterial strains. One hundred and eighteen strains of Proteus vulgaris isolated locally during 1966 were investigated. These strains and an additional 44 P. vulgaris strains were used as indicators. Bacteriocins were also tested against 44 newly isolated strains of P. mirabilis and large numbers of strains of other species of the family Enterobacteriaceae. All the Proteus strains differ from one another in the sense that when matched on nutrient agar a line of demarcation is present between their swarms (Dienes, 1946). Strains were maintained on agar at 4°. Cultures were incubated at 25°.

Ultraviolet-light source. A 30 w Hanovia sterilamp (wavelength 2537 Å) was used from a distance of 25 cm.

Detection of bacteriocins. A modification of the method of Abbott & Shannon (1958) was used. Cultures were streaked on MacConkey agar, incubated for 7 hr and irradiated for 4 min. The plates were incubated for 16 hr in the dark before the chloroform treatment and cross-streaking with indicator strains. Serial transmissibility of the killing effect was tested by the transfer of inhibitory areas to broth. The broth was shaken, sterilized with chloroform and spotted on a lawn of the indicator organism.

Production of bacteriocins in fluid medium. Bacteriocinogenic cultures were grown overnight in 50 ml. broth, diluted with 100 ml. broth and incubated for 10 min. Ten ml. volumes in Petri dishes were irradiated for 4 min. and incubated in the dark for 7 hr. At 30 min. intervals the optical density at 600 mμ was determined and samples were centrifuged at 6037 g for 30 min. and the supernatant fluids assayed.

Assay of bacteriocins. Activity in fluid medium was assayed on SS-agar by a spotting technique (Coetzee, 1967). The highest inhibitory dilution expresses the titre. The bactericidal property was tested by subculture in broth of clear areas of inhibition.

Concentration and purification of bacteriocins. Bacteriocins were precipitated with 40 % (w/v) ammonium sulphate, centrifuged at 6037 g for 30 min. and dissolved in 0.1 N-ammonium acetate (pH 7.2). Purification was by differential centrifugation at cycles of 6037 g and 54333 g for 30 min. and 180 min. respectively.

Agar electrophoresis was by the method of Maré, Coetzee & de Klerk (1964).

Diffusibility of bacteriocins. Bacteriocin in a sealed hole in MacConkey agar was kept at 4° for 18 hr. The extent of diffusion was measured by the application of an indicator organism (Coetzee, 1967).
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*Action of trypsin on bacteriocins.* Bacteriocins were incubated with 0.5 mg./ml. crystalline trypsin (British Drug Houses Ltd. (BDH)) in 200 mM-sodium phosphate buffer (pH 7.5) for 3 hr and titres determined. In control experiments the trypsin was omitted.

*Heat sensitivity.* Bacteriocins were heated from 50° to 70° in a water bath for 30 min. and samples assayed.

*Electron microscopy.* Samples were suspended in 0.1 N-ammonium acetate (pH 7.2) and negatively stained with neutral potassium phosphotungstate (Brenner & Horne, 1959). They were mounted on carbon support films by a spreading technique (Bradley, 1962) and examined in a Philips EM 200 electron microscope.

*Contraction of phage-tail-like structures.* Purified bacteriocin was treated with 3% (v/v) H₂O₂ and 10% (v/v) ethanol (Kellenberger & Arber, 1955). Samples were taken at minute intervals and diluted with 0.1 N-ammonium acetate. Bacteriocin was sedimented at 54,333 g for 3 hr, resuspended in 0.1 N-ammonium acetate (pH 7.2) and assayed. The samples were also mounted for electron microscopy. Controls were untreated bacteriocins.

*Adsorption of bacteriocins.* Indicator organisms were sedimented from 10 ml. overnight broth cultures and resuspended in 0.5 ml. of purified bacteriocin or bacteriocin treated with H₂O₂ and ethanol. The mixtures were incubated for 10 min., centrifuged at 6037 g for 30 min. and the supernatant fluids assayed and examined in the electron microscope. The sedimented indicator strains were mounted for electron microscopy. Controls with non-susceptible organisms were prepared concurrently.

*Protein estimation.* This was done by the method of Lowry et al. (1951) with crystalline bovine albumin (Armour Pharmaceutical Co., Kankakee, Ill., U.S.A.) as standard.

*Estimation of nucleic acids.* Purified bacteriocin (titre 1/4000) was extracted twice with 0.5 N-perchloric acid for 15 min. at 70° (Burton, 1956). DNA in the clear extract was estimated with the diphenylamine method (Burton, 1956) with 2-deoxy-D-ribose (BDH) as standard and RNA was estimated by the method of Dische (1953) with D(-) ribose (BDH) as standard. Values were corrected for the contribution of interfering carbohydrates.

*Carbohydrate estimation.* Total sugars were determined by the anthrone method of Scott & Melvin (1953) with glucose as standard.

**RESULTS**

*Incidence of bacteriocinogeny*

Seventy of the strains produced areas of inhibition on one or more of the *Proteus vulgaris* indicator cross-streaks. Only 3 of these inhibitory areas were serially transmissible and formed plaques on indicator organisms. Coetzee & Sacks (1960) found 4 of 7 strains of *P. vulgaris* to be lysogenic. Cradock-Watson (1965) reported 61% of 229 strains of *P. mirabilis* to be bacteriocinogenic and we found 67 of 118 (57%) *P. vulgaris* strains to be bacteriocinogenic. None of the bacteriocins inhibited non-Proteus species but all inhibited one or more strains of *P. mirabilis*. Nine bacteriocins had identical ranges of activity; 30 bacteriocins with different ranges of activity were chosen for further study. These bacteriocins inhibited from 5 to 87 *P. vulgaris* indicators and 4 to all 44 *P. mirabilis* strains.
**Production of bacteriocin in fluid medium**

All strains yielded inhibitory titres of about 1/100. Production of bacteriocin was associated with some cell lysis. Bacteriocins killed sensitive organisms since subcultures of clear areas of inhibition failed to show growth.

**Concentration.** Activity could be sedimented by high-speed centrifugation and precipitated by 40% (w/v) ammonium sulphate. There was no activity in supernatant fluids. Hundredfold concentration was achieved by these methods.

**Properties of bacteriocins**

**Heat sensitivity.** Bacteriocins with titres of 1/10,000 were completely inactivated at 60°C for 30 min.

**Diffusibility.** All 30 bacteriocins diffused about 1 cm. from the hole in agar during 18 hr. Phage 107/69 (Coetzee, de Klerk & Smit, 1967) tested under similar conditions did not diffuse.

**Electrophoretic mobility.** All the bacteriocins were immobile on electrophoresis under conditions where Proteus morganii bacteriocin MR 336 (Coetzee, 1967 and unpublished) moved 6 cm. towards the cathode.

**Susceptibility to trypsin.** Bacteriocins 50 and 116 were tested; titres of both were reduced 100-fold.

**Fine structure.** Electron microscopy of purified bacteriocins revealed masses of structures resembling sheathed phage tails (Pl. 1, fig. 1). Some bacterial debris was usually present. In 2 of the preparations occasional phage-like particles were also seen. Those in bacteriocin 35 (Pl. 2, fig. 2) closely resembled Proteus mirabilis phage 13 vir. while the phage-like particles in bacteriocin 71 (Pl. 2, fig. 3) looked like P. rettgeri phage 7476/322 (Prozesky, de Klerk, & Coetzee, 1965). No isolated phage-head-like objects were detected. Most of the tail-like structures were completely sheathed while in a few the sheaths were contracted and revealed hollow cores. The sheaths could be made to contract with H₂O₂ and ethanol and the titre of such preparations was zero (Pl. 2, fig. 4). 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 (Pl. 1, fig. 1). This projection appeared hollow (Pl. 2, fig. 5). The averages of 6 to 10 measurements of the tail-like structures of all 30 bacteriocins were identical, 1280 Å in length and 180 Å in width. Contracted sheaths were hollow cylinders 560 Å x 200 Å. The cores had an external diameter of 70 Å. Diagonal cross-striations suggestive of helical symmetry could be seen for short distances on some of the extended sheaths (Pl. 2, fig. 5). Small disc-like objects observed by Ishii et al. (1965), Bradley & Dewar (1966) and Takeya et al. (1967) which probably represented segments of cores were not a feature of untreated preparations. The contracted form of the structures was similar to the particles liberated by the P. mirabilis strains of van Iterson et al. (1967). The pyocins described by Ishii et al. (1965) and Bradley & Dewar (1966) were slightly smaller than the structures seen here and these workers also described tail fibres and base plates. In only 5 preparations, nos. 55, 60, 61, 62, 105, did the direct relationship between the number of structures and titre not hold. These preparations contained numerous fully sheathed tails but biological activity was low. Five (no. 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 strains and
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examined in the electron microscope. These 5 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 (Pl. 3, fig. 6). 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 have indicated that triggered tails do not adsorb.

**Chemical analysis of tail-like structures.** The composition (μg./ml.) of bacteriocin 45 with a titre of 1/4000 was protein, 640; DNA-phosphorus 0.0; RNA-phosphorus 0.48; total sugars, 412. The presence of sugars may be ascribed to bacterial cell walls which usually contaminated preparations of bacteriocin and the presence of RNA may have been due to bacterial ribosomes.

**DISCUSSION**

During purification and concentration of the tail-like structures killing activity was always associated with fully sheathed forms of the particles. With few exceptions the impression was that the number of uncontracted particles bore a direct relation to the activity of preparations. The 5 exceptions may be due to a lack of more susceptible indicators, although no. 55, 60 and 61 attacked 48, 48 and 62 *Proteus vulgaris* strains respectively and the range of the remaining 2 was only slightly smaller.

The structures described were occasionally associated with phage-like particles which may have been temperate phages (Bradley & Dewar, 1966). They also closely resembled the sheathed contractile tail of phage 107/69, a temperate transducing phage of *P. vulgaris* (Coetzee et al. 1967). Taubeneck (1963) considered the phage-tail-like structures liberated by *P. mirabilis* strain 52 to be the product of defective lysogeny. These particles lacked DNA and upon adsorption the sheaths contracted but the cores did not penetrate the cell wall; they projected outwards beyond the sheaths. Shadow-cast preparations were presented and detailed comparison with structures described here is not possible. The fully sheathed structures of *P. vulgaris* adsorbed to bacteria-like phage tails. The visible portion of the core of particles with uncontracted sheaths appeared hollow. These structures did not contain DNA and may have been empty sheathed phage tails liberated by defective lysogenic *P. vulgaris* strains. They possibly killed bacteria like the ghosts of T₂ phage (Herriot, 1951) but the problem still remains how a defective prophage, which is temperate by definition, acquires a lethal tail (Taubeneck, 1963; Seaman, Tarmy & Marmur, 1964; Stickler & Tucker 1967). Kellenberger & Séchaud (1957) demonstrated that core-like structures of phage T₂ adsorbed to but did not kill *Escherichia coli* while similar structures derived from T₄ did not adsorb. Triggered *P. vulgaris* tail-like structures also did not adsorb. Arber & Kellenberger (1958) demonstrated that phage-tail-like structures produced by defective lysogenic strains of *E. coli* K₁₂ adsorbed to a strain c₁₀.

Many phages isolated on *Proteus mirabilis* strains also lyse *P. vulgaris* strains and *vice versa* (Coetzee, 1963). This is one of the facts favouring a close relationship between
these two groups (Smit & Coetzee, 1967). The finding that the P. vulgaris structures described here also kill strains of P. mirabilis and no other species supports this argument.

The 5 strains (nos. 12, 20, 26, 58, 63) with no killing effect which do not produce phage-like structures still form a Dienes demarcation line (Dienes, 1946) between their swarms on agar. This eliminates these structures as a possible cause of the Dienes phenomenon (see Hughes, 1957).

Bacteriocins range from molecules with a sedimentation coefficient of 2.85 for a Lactobacillus fermenti bacteriocin (de Klerk, & Smit, 1967) and 3.6 for colicin E2 (Reeves, 1965) through the phage-tail-like components of pyocins (Ishii et al. 1965) to the phage-like colicins 15 and H (Endo et al. 1965; Bradley & Dewar, 1966). Bradley & Dewar (1966) suggested that the 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.

A striking feature of this work is that all of 30 killing strains of Proteus vulgaris produced 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 67 of the killer strains produce these tail-like structures. Although the latter are morphologically identical they differ in biological activity. If they are accepted as products of defective lysogeny it is possible that the latter state (Campbell, 1961; Neubauer, 1967) has arisen by selection only for the favourable genes acquired by transduction (Coetzee, Smit & Prozesky, 1966; Coetzee et al. 1967) or conversion (Coetzee, 1961) in originally lysogenic Proteus.

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REFERENCES


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EXPLANATION OF PLATES

All preparations in ammonium acetate and phosphotungstate.

PLATE 1

Fig. 1. Phage-tail-like particles of bacteriocin 45. Arrows indicate projecting cores. The projections appear hollow.

PLATE 2

Fig. 2. Phage-tail-like structures of bacteriocin 35 and a phage-like structure.

Fig. 3. Phage-like structure from bacteriocin 71.

Fig. 4. Phage-tail-like structures of bacteriocin 11 treated with H2O2 and ethanol. This shows contracted sheaths round hollow cores and empty sheaths.

Fig. 5. Phage-tail-like structure of bacteriocin 35 with uncontracted sheath and projecting tail core. The projection appears hollow. Arrows indicate diagonal cross-striations.

PLATE 3

Fig. 6. Portion of a cell of Proteus vulgaris strain 10 with bacteriocin 37 around the organism. Fully sheathed and contracted forms are visible between the pili. A fragment of a flagellum is also present.