The Structure of Phage-Like Objects Associated with Non-Induced Bacteriocinogenic Bacteria

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SUMMARY

Phage-like objects obtained from bacteriocinogenic strains of *Escherichia coli*, *Pseudomonas aeruginosa* and *Listeria monocytogenes* by the differential centrifugation of culture fluids were examined in the electron microscope. Particles with small heads and contractile tails were found associated with strains of *E. coli*. The three *P. aeruginosa* strains all produced numerous headless contractile tails in broth cultures together with a very few phage-like objects with flexible non-contractile tails. The headless tails possessed cores which appeared both solid and hollow in the electron microscope, indicating the presence of some unidentified substance, perhaps nucleic acid. The only type of particle found associated with *L. monocytogenes* was a large bacteriophage with a complex contractile tail. The structure of these particles is described and discussed, and it is concluded that some of them are the bactericidal principles of spontaneously released bacteriocins.

INTRODUCTION

The bacteriocins are a group of highly specific antibiotics, of which only a few have been studied in detail while a great many have been isolated and classified (for recent review see Reeves, 1965). The individual bacteriocin 'species' are usually referred to by a derivation of the Linnaean specific name of the organism which produces them. For example, the bacteriocins of *Escherichia coli* and *Listeria monocytogenes* are called colicins and monocins respectively. The characteristic physiological property of bacteriocins is their ability to kill a sensitive cell, but not to multiply within it. They are perpetuated by genetic information within the host in the form of plasmids, vegetative particles released in the same way as temperate bacteriophages; they may be spontaneously produced in host cell cultures or may have to be induced.

Comparatively few attempts have been made to study the morphology of bacteriocins with the electron microscope. Kellenberger & Kellenberger (1956) showed phage-like particles associated with a colicin-producing strain of *E. coli*. More recently Sandoval, Reilly & Tandler (1965) and Endo, Ayabe, Amako & Takeya (1965) found that colicinogenic *E. coli* wt15 produced similar particles which were identified as the bacteriolytic principle (Colicin 15). These were small-headed phage-like particles with contractile tails and were apparently identical to the virulent coliphage ϕ 1 (Bradley, 1963). Electron microscopy has also shown that the purified pyocin produced by *Pseudomonas aeruginosa* strain R consisted of short rods

identical to headless tails of contractile bacteriophages (Ishii, Nishi & Egami, 1965); the rods were found both contracted and extended.

A few phage-like particles which are apparently identical to bacteriocins, but which have not been classified as such, have been isolated for several *Bacillus* species (Seaman, Tarmy & Marmur, 1964; Ionesco, Ryter & Schaeffer, 1964; Stickler, Tucker & Kay, 1965; Bradley, 1965*a*). These-so-called 'killer' particles were described as defective temperate bacteriophages lacking the ability to multiply intracellularly but able to lyse a sensitive cell. They are thus physiologically identical to bacteriocins. Those studied in greatest detail in the electron microscope were of similar basic morphology to bacteriophages with contractile tails, but had unusually small heads, only about 300 Å in size. More conventional forms with contractile and non-contractile tails were also encountered.

From these observations it seems probable that a substantial number of bacteriocins are phage-like particles or phage components. The present communication describes the results of an electron microscopic examination of these particles associated with bacteriocinogenic strains of *Escherichia coli*, *Pseudomonas aeruginosa* and *Listeria monocytogenes*.

METHODS

Host bacteria. A number of bacteriocinogenic strains of the species mentioned above were kindly supplied by Dr Y. Hamon, Institut Pasteur, Paris, and these are listed in Table 1. In addition, indicator strains were supplied for bactericidal activity tests. These were *Escherichia coli* K12, *Pseudomonas aeruginosa* P1050, and *Listeria monocytogenes* 23.

Table 1. *Spot tests for bactericidal activity*

Species	Strain	Bacteriocin no.	Spot test of bacteria	Spot test of extract
<i>Escherichia coli</i>	CL10	H	+	+
<i>E. coli</i>	K235	K	+	—
<i>E. coli</i>	167	I	+	—
<i>Pseudomonas aeruginosa</i>	KR35	—	±	—
<i>P. aeruginosa</i>	ST-6	—	+	—
<i>P. aeruginosa</i>	TTC	—	±	—
<i>Listeria monocytogenes</i>	1896 x	—	+	+

A + sign denotes a clear area around bacteria or a clear plaque produced by a culture extract. A ± sign denotes a veiled surround or plaque. A — sign denotes no activity.

Preparation of bacterial culture extracts. As has already been stated, bacteriocins or temperate phages are usually present in detectable numbers in cultures of the host bacterium. Suitable extracts should therefore contain sufficient particles for electron microscopy after concentration; accordingly, two simple methods of preparation were used.

In the first, double agar layer plates (Adams, 1959) of the bacteria were prepared using Oxoid nutrient broth medium at double strength. After incubation overnight at 37°, 2–3 ml. of neutral 0.1 M-ammonium acetate solution was poured onto the plate, which was allowed to stand for 3–4 hr at room temperature with occasional agitation. The extract obtained was then poured off and centrifuged at 2000–3000 *g*

for 20 min. to remove bacteria. The supernatant was centrifuged at 15,000 *g* for 3–4 hr to sediment any phage-like particles present. The pellet obtained was re-suspended in 0.1 ml. of neutral 0.1 M-ammonium acetate and centrifuged again at 2000–3000 *g* for 20 min. to remove any remaining bacteria. The resulting supernatant suspension was then ready for electron microscopy.

In the second method, a 10 ml. broth culture of host bacteria was incubated for about 5 hr in a shaker at 37°. Bacteria were sedimented at 2000–3000 *g* for 20 min. In some cases the culture was shaken with chloroform before centrifugation. The supernatant was then centrifuged at 15,000 *g* for 3–4 hr and the pellet suspended in ammonium acetate solution as above. The latter method was found most satisfactory.

Spot tests for bacteriocin activity. Both bacteria and concentrated culture extracts were tested by the spot test (Bradley, 1964) for activity against sensitive indicators. This was carried out by placing a loopful of bacteria or extract on the surface of a double agar layer plate of the indicator strain. After incubation, local clearing indicated bacteriocin activity.

Electron microscopy. Negative staining with potassium phosphotungstate (PTA) was used throughout. The suspension in ammonium acetate prepared as above was mixed with an equal volume of neutral 2% (w/v) PTA solution. A carbon-coated grid was then touched on to the surface of the mixture; all but a thin film of liquid was removed from the grid with the edge of a filter paper. When dry, the specimen was ready for electron microscopy, in this case in a Siemens Elmiskop I.

One of the micrographs was printed by rotation (Markham, Frey & Hills, 1963) as follows. A normal print was accurately centred on a gramophone turntable and fixed with adhesive tape. The turntable was then placed under an enlarger containing the original negative, and the projected image of the object of interest was carefully mated with that on the print. The enlarger was then switched off and a piece of printing paper fixed to the turntable on top of the print already there. The enlarger was then switched on for six periods of time, each equal to one sixth of the total exposure required for a normal print; the turntable of the gramophone was rotated by exactly one-sixth of a revolution between each exposure. On developing, a rotation print was obtained in which the information from each 60° segment of the image was integrated with the rest. It was found that some contrast was usually lost by this procedure, and that it was best to use an extra hard grade of paper.

RESULTS

Spot tests for bacteriocin activity

The results of spot tests of both bacteria and the concentrated culture extracts used for electron microscopy are given in Table 1. Strains producing colicins gave most activity, with clear areas more than 1 mm. wide with sharply defined edges round colonies. Those producing pyocins gave little activity, with a 1 mm. surround of usually veiled appearance and a diffuse edge; the exception to this was *Pseudomonas aeruginosa* strain ST-6. Strains of *Listeria monocytogenes* producing monocins (monocinogenic) formed a narrow (1 mm. or less) area which was clear. It can be seen from Table 1 that only two culture extracts were active against appropriate sensitive indicators; the significance of this will be discussed below.

Electron microscopy of culture extracts

Escherichia coli strain *CL10*. Extracts of plate cultures contained numerous headless contracted tails. Better results were obtained from broth culture extracts, however, which contained many phage-like particles with heads. In Pl. 1, fig. 1, an example can be seen to be a conventional contractile particle similar to coliphages $\phi 2$ (Bradley & Kay, 1960) and E1 (Bradley, 1963), a form which is common in nature. The head is 650 Å in size and the tail about 1800 Å long. In Pl. 1, fig. 1, the head is filled with what is presumably deoxyribonucleic acid (DNA), and the sheath is extended. There is a thin base-plate, but no obvious striations on the sheath. Both plate and broth culture extracts contained a very small number of larger particles, one of which is shown in Pl. 1, fig. 2. It is a contractile type with a 950 Å head and 2800 Å tail; the one shown has a full head and contracted sheath. Others had empty heads.

Escherichia coli strains *K235* and *167*. There were very few phage-like particles associated with these strains; only one or two could be found in each electron microscope grid square. They are illustrated in Pl. 1, figs. 3, 4, and can be seen to be basically similar to the *CL10* particles. The heads are about the same size (650 Å) but the tail from strain *K235* is rather longer (2000 Å). That from strain *167* is, however, only 1500 Å long, but it has a small collar at the top of the contracted sheath.

Pseudomonas aeruginosa strains *KR35* and *ST-6*. Strain *KR 35* yielded a few phage-like particles with long non-contractile tails. One with a full head is shown in Pl. 1, fig. 5, and an empty one in Pl. 1, fig. 6. The head is about 700 Å in size and exhibits a six-sided outline; the 1800 Å-long tail has no obvious striations. These resemble the *Pseudomonas* phage *pc* described elsewhere (Bradley & Kay, 1960). In addition strain *KR 35* contained a small number of headless contracted tails. That shown in Pl. 1, fig. 7, is 1400 Å long; it has a solid core and a contracted sheath with what appear to be fibres attached to it. An extract of strain *ST-6* contained no intact phage-like particles but a few headless tails in both a contracted and extended state. In Pl. 1, fig. 8, the contracted tail (length 1400 Å) is hollow. Pl. 1, fig. 9, shows an extended tail, also 1400 Å long, and with marked striations of periodicity of about 37 Å. The tail appears to be hollow for the top 225 Å of its length, but solid for the rest. Both these micrographs also show small discs or perhaps spheres about 120 Å in diameter. Those at the bottom of Pl. 1, fig. 9, suggest a hexagonal outline.

Pseudomonas aeruginosa strain *RTC*. Like *KR35* and *ST-6* this strain had headless tails associated with it, but in much larger numbers. This allowed a rather more detailed study. A group of particles is shown in Pl. 2, fig. 10. It can be seen that they are identical to those already described. Their average length is also 1400 Å. They seem to be solid except for the top 200 Å; the two contracted tails in Pl. 2, fig. 10, are also solid. There is an isolated core with a suggestion of periodicity on it at the bottom centre of the micrograph; this is empty and is readily distinguished from the full or solid cores of the contracted tails. More detail is seen in Pl. 3, fig. 11: striations with a periodicity of about 37 Å and the hollow portion of the core, particularly in the bottom right particle. In Pl. 3, fig. 12, the difference between empty and full cores is shown more clearly. The top particle has a full one, and the other two empty ones; this is a particularly important observation which will be discussed below

In a very few cases (Pl. 3, fig. 13) the helical nature of the sheath was visible. It is a coarse helix similar to that found on T-even coliphages (Bradley, 1963). In some cases the core had slipped out of the contracted sheath leaving a short tube (Pl. 3, fig. 14); two are lying end-to-end in this micrograph. The hole of this tube is 90 Å in diameter, but the cores themselves are only 60 Å wide. As suggested from Pl. 3, fig. 12, there is a slight gap between the core and the tube wall of the extended sheath. The diameter of the extended sheath is 180 Å and that of the contracted sheath, 225 Å.

In addition to headless tails, numerous long rod-shaped objects were found (Pl. 3, fig. 15). At first these might be thought to bear no relationship to the particles just described, but their diameter is 225 Å, like the contracted sheaths, and they resemble the polysheath of T-even coliphages (Poglazov & Tikhonenko, 1963; Kellenberger & Boy de la Tour, 1964).

Listeria monocytogenes strain 1896 x. An extract of a broth culture of this strain provided a rich suspension of phage-like particles (Pl. 4, fig. 16). As with *Pseudomonas aeruginosa* these were largely headless tails. However, a few similar tails attached to heads (Pl. 5, figs. 17, 18) were also found and there were numerous tailless heads (Pl. 5, fig. 19) in the preparation. All the heads, whether attached or detached, were empty; they had an average diameter of 1100 Å. The total tail length of 2400 Å can be seen in Pl. 4, fig. 16, to consist of about 1600 Å of sheath with 800 Å of core projecting from the end. The sheath diameter is 200 Å and that of the core about 75 Å. In Pl. 4, fig. 16, there is also a smaller head (600 Å) with a longer core (2700 Å) attached. The sheaths in Pl. 4, fig. 16, have a very fine capsomere structure whose organization is not apparent, though four parallel axial lines are very obvious. The arrangement of the tail tip is clearer in Pl. 5, figs. 17, 18. There is a distinct base-plate with two prongs at its edge (these are shown as fine fibres in Pl. 5, fig. 21) and a wedge-shaped structure below the base-plate. The cores in the first two particles (Pl. 5, figs. 17, 18) are solid, but in the headless ones they are hollow. The appearance of the sheath is also different on particles with heads: neither the fine subunits nor the longitudinal lines can be seen, though the negative staining and preservation was equally good.

In Pl. 5, fig. 20, the base-plate with its fibres is partially detached from a coreless sheath. This preparation also contained some hexagonal plates (Pl. 5, fig. 22) which can be correlated with the base-plate in Pl. 5, fig. 20. They had obviously become detached from the sheaths. The base-plate in Pl. 5, fig. 22, is clearly of six-fold rotational symmetry and may have some morphological subunits on its rim. The use of the rotational printing procedure (Markham *et al.* 1963) clarifies the whole structure; Pl. 5, fig. 23, has been rotated by 120° between each exposure and Pl. 5, fig. 24, by 60°, i.e. three and six exposures per revolution respectively. There is a progressive improvement in definition in each case clearly showing the shape of the fibres, and also revealing the presence of six subunits slightly to one side of the centre of each edge of the hexagon. Since these are more electron-transparent than the rest of the plate they must consist of a greater vertical thickness of protein.

In Pl. 6, figs. 25, 26, isolated cores are shown in the form of the usual hollow tubes. That in Pl. 6, fig. 25, appears to be pulled out from the sheath; beside it there are five small star-shaped objects. Their origin is shown in Pl. 7, fig. 28, where the sheaths have been partly degraded into individual capsomeres. These minute

objects are hexagonal plates with a subunit at each apex, giving them a star-like appearance; they measure about 100 Å across.

A tail which has lost its base-plate is shown in Pl. 6, fig. 27; its significance will be discussed below.

DISCUSSION

Phage-like particles from Escherichia coli. All three strains of *E. coli* produced highly active colicins (see Table 1) but only one of the extracts prepared for electron microscopy possessed any killing ability. This strain (CL10) produced an abundance of phage-like particles, whereas the other two contained barely detectable numbers. Bearing in mind the low centrifugation speed used to sediment the particles it would seem that the CL10 particles represent colicin H but that those of the other two strains do not represent the bactericidal principle. This is of importance since Sandoval, *et al.* (1965) showed particles associated with *E. coli* WT15, similar to those of CL10, which were almost certainly the lytic principle. One deduces from this that some colicins are defective bacteriophages, as suggested by Sandoval *et al.* (1965) for colicin 15, and some are much smaller, not being sedimented by the 15,000 *g* used in the present investigation. As Reeves (1965) pointed out, colicin CA42-E₂ has a sedimentation coefficient of only 3.6S meaning that it will be quite different in form and function from colicins 15 and H. The suggestion of Ivanovics (1962) and Fredericq (1963) that colicins are the products of defective lysogeny appears, therefore, to be partly confirmed. It does seem, however, that there may be two completely different types within this group of bacteriocins.

Although there is a basic similarity between the particles of colicin 15 illustrated by Sandoval *et al.* (1965) and those of colicin H shown here, their preparation of colicin 15 contained many empty tail-less heads as well as a substantial number of intact phage-like particles. Since the function of the particles in both cases is now defined, they may be considered physiologically similar to the so-called 'killers' of *Bacillus subtilis* mentioned above (see Introduction).

It is interesting to compare the size of the CL10 particle with that of coliphage E1 (Bradley, 1963) which is morphologically similar. The heads are the same size (650 Å) but the tail of colicin CL10 is 1800 Å long compared with 1150 Å for that of the phage.

Particles from Pseudomonas aeruginosa. The headless tails found associated with pyocinogenic strains of *P. aeruginosa* all looked the same and were identical to the purified pyocin described by Ishii *et al.* (1965). As can be seen from Table 1, the spot tests were not conclusive, but because of the similarity to a properly purified pyocin, it is certain that these headless tails represent the lytic principle.

The pyocins have been well illustrated in order to permit a detailed comparison with the one described by Ishii *et al.* (1965). There is no necessity to go into details since it is abundantly clear that the structure of all four pyocins is identical. The only possible difference is in the length of the particles; those described here are 1400 Å long, and those of Ishii *et al.* (1965) were 1200 Å long. This difference is not considered significant. One or two points, however, have been omitted by these authors.

The most important and significant is the presence of both solid and hollow cores in contracted tails; this indicates unequivocally that something is present in the

tube. The conclusion is supported by the observation that the top 200 Å of extended tails is empty; the rest is presumably filled with some undefined substance. A somewhat analogous observation was made on the virulent pseudomonas phage SLO by Bradley (1965*b*); here a single empty contractile sheath in the extended state with a 20 Å hole down the centre was found. The obvious and most likely deduction is that the material in the core is a short length of nucleic acid. Also supporting this is the fact that the nucleic acid strand of tailed bacteriophages extends from the head to the tail tip (see Bradley, 1965*b*). Since all known phages with contractile tails have deoxyribonucleic acid (DNA) as their genetic material, it is reasonable to suppose that the strand within these headless tails is of the same kind. Since the core is capable of being emptied and the tail is of a contractile nature, its contents are doubtless injected into the bacterium when the pyocin adsorbs to the cell surface. This short length of DNA could then misdirect the bacterial metabolism and so cause the death of the cell. It is not contended that this mode of action is anything more than a theory suggested by the presence of a structure within the tail tube. However, such a hypothesis could be confirmed by nucleic acid estimations and perhaps by suitable radioactive tracer experiments.

The nature of the minute disc-like objects in Pl. 1, figs. 8, 9, is not known. These are rather larger than those found by Ishii, *et al.* (1965), which seemed to be segments of cores. With a diameter of 120 Å they are also too small to be segments of the extended sheath. The non-contractile phage-like particles found with KR35 are probably temperate bacteriophages.

Phage-like particles from Listeria monocytogenes. No monocins have hitherto been examined in the electron microscope. In view of the biological activity of the electron microscope preparation, it seems that the particles observed here are the bactericidal principle. While basically of a contractile nature, no particles with obviously extended sheaths could be found. However, the sheaths of the particles in Pl. 5, figs. 17, 18, differ in capsomere structure from the others shown, though the sheath length is the same. The cores are solid, whereas the headless tails have empty ones, suggesting that the former are viable and the latter are not. In this case the head would be naturally empty and would play no part in the killing process. Such a particle could represent an intermediate stage between the colicins and the bacillus 'killers' with their full heads, and the pyocins with their headless tails.

Structurally the particles are of unusual interest mainly because it is possible to resolve the shape of the individual tail capsomeres. Two forms have been tentatively observed for those of conventional phages; short tubes and 'globular' subunits. There is no reason why the star-shaped objects should not represent short tubes seen end-on. Unfortunately there is insufficient information in the electron micrographs to suggest a model for their arrangement, let alone any change in arrangement which may attend sheath contraction. Indeed, there is no evidence to suggest that contraction occurs in the conventional manner; from what one can see, there is merely a change in capsomere packing without a shortening of the sheath. If a morphologically similar virulent bacteriophage for *L. monocytogenes* were found, this point could probably be cleared up; those known are non-contractile (Sword & Pickett, 1961).

The tail without a base-plate shown in Pl. 6, fig. 27, is included because it bears a remarkable resemblance to the rhabdosomes or rod-shaped bodies found by

Correll & Lewin (1964) associated with the gliding organism *Saprospira grandis*. The sheath length of the latter is 1200 Å and that of the monocin is 1500 Å. The total length of each is about the same at 2700 Å. The inference is that the *S. grandis* rhabidosome is a particle arising from a degenerate contractile bacteriophage.

The nature of bacteriocins. While only a few bacteriocins have so far been examined in the electron microscope, it is clear that a knowledge of their structure is valuable in elucidating their true nature. From the results obtained above and those cited in the literature, it can be said of the colicins that two kinds exist: on the one hand there are phage-like particles, and on the other smaller as yet unidentified molecules which presumably act in a different way. With the pyocins the position is different. All four examples so far studied are identical and constitute what is essentially a unique type of bacteriolytic particle. Whether or not all pyocins are the same will only be discovered after an extensive survey. The monocin examined is again different and further study is also required to see whether it is typical of the monocins as a group. Some bacteriocins which have only been mentioned here are those specific to species of the genus *Bacillus*; these are the *Bacillus* 'killers'. A number of bacteriocins specific to *B. megaterium* (megacins) and *B. cereus* (cerecins) also exist (see Reeves, 1965) and it is obviously important to see whether or not they resemble bacteriophages. Above all it is clear that a wide range of morphological and physiological bacteriolytic particles exist in the bacteriocins, and both the general conception and classification of the group will clearly have to be revised in the light of future work.

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EXPLANATION OF PLATES

PLATE 1

- Fig. 1. Small particle from *Escherichia coli* CL10. $\times 180,000$.
- Fig. 2. Large particle from *E. coli* CL 10. $\times 180,000$.
- Fig. 3. Particle from *E. coli* K 235. $\times 180,000$.
- Fig. 4. Particle from *E. coli* 167. $\times 300,000$.
- Figs. 5, 6. Full and empty phage-like particles from *Pseudomonas aeruginosa* KR 35. $\times 180,000$.
- Fig. 7. Contracted headless tail from *P. aeruginosa* KR35. $\times 300,000$.
- Figs. 8, 9. Headless tails from *P. aeruginosa* ST-6. $\times 300,000$.

PLATE 2

- Fig. 10. Particles from *Pseudomonas aeruginosa* TTC showing contracted and extended sheaths and isolated core. $\times 200,000$.

PLATE 3

- Fig. 11. Particles from *Pseudomonas aeruginosa* TTC extended sheaths showing striations and tubular capsomeres. $\times 275,000$.
- Fig. 12. Contracted sheaths showing empty and full cores. $\times 275,000$.
- Fig. 13. Extended sheath showing coarse helix. $\times 275,000$.
- Fig. 14. Isolated contracted sheaths. $\times 275,000$.
- Fig. 15. Rod-shaped objects. $\times 165,000$.

PLATE 4

- Fig. 16. Particles from *Listeria monocytogenes*. $\times 200,000$.

PLATE 5

Particles from *Listeria monocytogenes*

- Figs. 17, 18. Particles with heads. $\times 280,000$.
- Fig. 19. Isolated head. $\times 280,000$.
- Fig. 20. Base-plate being detached from isolated sheath. $\times 280,000$.
- Fig. 21. Particle showing base-plate fibres. $\times 280,000$.
- Fig. 22. Base-plate. $\times 415,000$.
- Fig. 23. The same, printed by rotation at three exposures per revolution.
- Fig. 24. The same, printed by rotation at six exposures per revolution.

PLATE 6

Particles from *Listeria monocytogenes*. $\times 200,000$.

Fig. 25. Core with star-shaped capsomeres.

Fig. 26. Isolated cores.

Fig. 27. Sheath and core without base-plate.

PLATE 7

Fig. 28. Sheath capsomeres from *Listeria monocytogenes* particles. $\times 333,000$.

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