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R-Body-Producing Bacteria

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INTRODUCTION

R bodies are unusual bacterial inclusion bodies. They are highly insoluble protein ribbons, typically seen coiled into cylindrical structures within cells. It has been noted that R bodies unwind under certain conditions and are associated with toxicity. Until recently they have been known to occur only in certain species of bacteria that are obligate endosymbionts of paramecia. However, in the last 10 years, several free-living bacterial species that have the ability to produce R bodies have also been reported.

Five classes or types of R bodies have been described (87, 88). R-body types are distinguished on the basis of physical dimensions, morphology, and behavior in response to certain physical and chemical treatments (Fig. 1). The names of the R-body types are derived from the bacterium name or the strain number of the bacterium in which they were first discovered. Type 51 and type 7 R bodies were first seen in strains 51 and 7 of cytoplasmic kappa symbionts in *Paramecium* species, respectively (these strains are now recognized as different species: *Caedibacter taeniospiralis* 51 and *C. varicaedens* 7). Type Cc R bodies are found in the macronuclear kappa symbiont *C. caryophila*. Type Pt R bodies are produced by *Pseudomonas taeniospiralis*, and type Pa R bodies are synthesized in *Pseudomonas avenae*.

Maximum R-body size ranges from 0.8 μm wide and up to 30 μm long for the largest R-body types, type Cc and type Pa, to 0.25 μm wide and less than 10 μm long for the smallest R bodies, type Pt. Type 51 and type 7 R bodies are intermediate in width and length (0.5 μm and 10 to 20 μm, respectively). Other than size differences, morphological differences are generally restricted to whether the ribbon termini are tapered or blunt. The inner terminus (around which the ribbon coils) is tapered in all R bodies, but the outer terminus can be blunt, as for type 7 and type Cc R bodies, or tapered, as for type 51, type Pt, and type Pa R bodies. However, Pa R-body ribbons are considerably different from other types in that tapering begins at the ribbon center and continues outward in either direction so that the ribbon is very sharply tapered at the ends.

R bodies unroll in one of two ways. Types 7 and Pa R bodies unroll from the outside, and types 51, Cc, and Pt unroll from the inside in a telescopic fashion. Also, the stimulus that induces the ribbons to unroll differs for the various R-body types. Lowered pH, to below 6.5, is apparently required for types 51 and Pa R bodies to unroll (63, 72). Other factors, such as ionic strength and the presence or absence of certain ions, may also be important in unrolling. Types 7 and Pt R bodies do not respond to changes in pH but can be made to unroll by incubation for about 10 min at 70°C (31, 72). Unrolling of type 51 R bodies has been shown to be reversible. Rerolling of the type 51 R-body ribbon occurs when the pH is brought above 7.0.

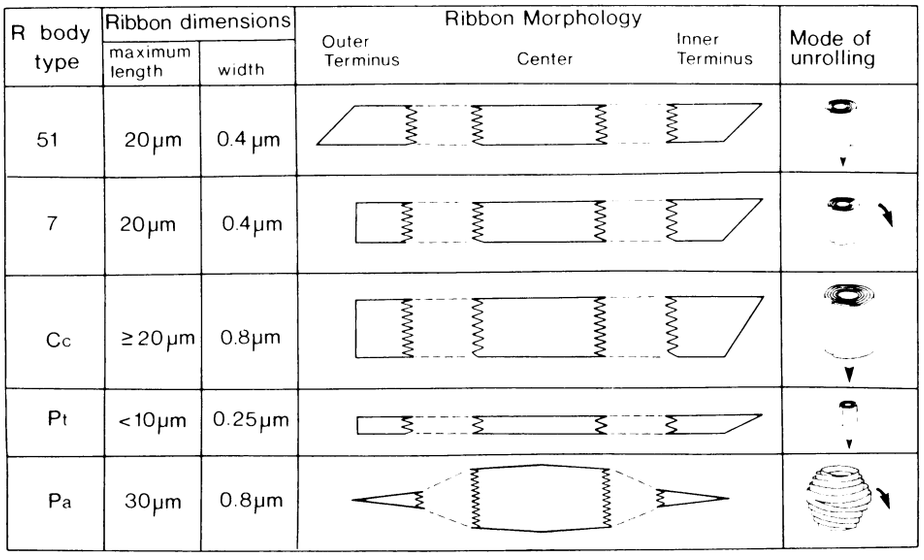


FIG. 1. Distinguishing features of different R-body types. The R bodies of strain EPS-5028 and *R. centenum* have not been described in detail and so are not included in any R body class. In the right-hand column, an arrow pointing down indicates unrolling from the inside outward. An arrow pointing to the side indicates unrolling from the outside. Note that the type Pa R body is drawn as a loose coil to better show its nature, although it is actually tightly coiled in its host cell. Included in the type 7 R-body class are the R bodies of *C. paraconjugatus*, which are smaller than other type 7 R bodies (about 5 μm long and 0.2 μm wide).

TABLE 1. R-body-producing bacteria

Species	Strain[a]	Collection site	Reference
Kappa particles			
C. taeniospiralis	51	Spencer, Ind.	8
	47	Berkeley, Calif.	22
	116	Bloomington, Ind.	80
	169	Morioka City, Japan	59
	298	Empire Range, Panama	80
	A30	Littlehampton, Australia	113
C. varicaedens	7	Pinehurst, N.C.	69
subsp. 7	249	Chipola River (U.S. 90), Fla.	80
	310	New Zealand	80
	576	Norwich, England	80
	1039	Lomonosov, USSR	80
	1041	Leningrad, USSR	80
	B1-166-1	Edinburgh, Scotland	80
C. varicaedens	562	Milan, Italy	8
subsp. 562	511	Figate, Scotland	80
	517	Gif, France	80
C. varicaedens subsp. 1038	1038	Syktykar, USSR	80
C. pseudomutans	51m1	Spencer, Ind.	22
C. paraconjugatus	570	Georgia, USSR	8
C. caryophila	C221	Münster, Federal Republic of Germany	95
	–[b]	Amiens, France	25
Free-living bacteria			
P. taeniospiralis	2K1	Barcelona, Spain	56
P. avenae	NCPPB-1001		125
Pseudomonas sp.[c]	EPS-5028	Barcelona, Spain	26
R. centenum	–[b]	Bloomington, Ind.	

[a] The first strain listed for each species is the type strain.
[b] –, No strain name.
[c] Strain EPS-5028 is presumed to be a pseudomonad (16), although it has not been officially classified as yet.

Only a few bacterial species are known to produce R bodies. The kappa symbionts of *Paramecium* species constitute the genus *Caedibacter* (75). Members of this genus are characterized by their ability to produce R bodies, their ability to produce a toxin, and their obligate symbiotic relationship with paramecia. Five species of kappa have been described: *C. taeniospiralis*, *C. varicaedens*, *C. pseudomutans*, *C. paraconjugatus*, and the recently described *C. caryophila* (95). Several free-living strains of R-body-producing bacteria have been recently reported, including two species of *Pseudomonas*, *Pseudomonas avenae*, a plant pathogen (125), and *Pseudomonas taeniospiralis*, a hydrogen-oxidizing soil bacterium (54), and *Rhodospirillum centenum*, a photosynthetic bacterium (H. Gest, personal communication). A fourth free-living, R-body-containing bacterium (strain EPS-5028), discovered in soil collected in Barcelona, Spain, has yet to be classified (26). The R-body-producing bacteria are listed in Table 1. Table 2 presents distinguishing characteristics of R-body-producing bacteria.

It has been over 30 years since R bodies were first discovered in kappa symbionts of killer paramecia. Initially, R bodies were of interest because their presence in the kappa symbionts of paramecia correlated with a killing trait. Research on kappa particles and the R bodies they produce has been historically important because it has served to focus attention on cytoplasmic inheritance, endosymbiosis, and questions of heredity versus infection. Several excellent reviews written in the early 1970s have considered the nature of *Paramecium* endosymbionts and the relationship of R bodies to the killing trait in paramecia (28, 76, 104). In the last 10 years, research in this field has advanced considerably, warranting another review of the literature. Research has now entered the arena of molecular biology, with the cloning of the R-body-encoding deoxyribonucleic acid (DNA) sequences for type 51 R bodies (89). Also, the discoveries of R-body-producing free-living bacteria (26, 54, 125) have widened the scope of R-body research and raised new questions about the evolution of R-body-producing bacteria and the relationship of R bodies with toxicity. New advances and discoveries allow previous data and observations to be reinterpreted in a new light.

R-BODY-CONTAINING *PARAMECIUM* ENDOSYMBIONTS

Historical Background

The killer trait discovered. In 1938, Sonneborn (108) discovered the existence of killer and sensitive paramecia. When individuals from a sensitive strain and members of a killer strain are placed together in a culture dish, the sensi-

TABLE 2. Distinguishing characteristics of R-body-producing bacteria

Species	Host (site of endosymbiosis)	Extrachromosomal elements	R-body type	Killing
C. taeniospiralis	*P. tetraurelia* (cytoplasm)	Plasmid	51	Hump killing[a]
C. varicaedens	*P. biaurelia* (cytoplasm)	Phage	7	Various types
C. pseudomutans	*P. tetraurelia* (cytoplasm)	Phage	7	Spin killing
C. paraconjugatus	*P. biaurelia* (cytoplasm)	Phage	7	Mate killing
C. caryophila	*P. caudatum* (macronucleus)	Phage	Cc	Paralysis[b]
P. taeniospiralis	Free living	Phage[c]	Pt	Sensitive paramecia killed[d]
P. avenae	Free living	ND[e]	Pa	Leaf blight[f]
Strain EPS-5028	Free living	Phage[c]	Unclassified	ND
R. centenum	Free living	ND	Unclassified	ND

[a] The target organisms for *Caedibacter* species are sensitive strains of paramecia.
[b] The exact form of killing has not yet been determined, but preliminary observations (Quackenbush, unpublished) suggest that killing may occur by paralysis.
[c] There is evidence that these species harbor defective prophages (25, 51), but it is not yet certain that R bodies in these species are encoded by a phage genome.
[d] *P. taeniospiralis* kills sensitive paramecia (49), but death is not characterized by distinct prelethal symptoms, nor has killing been linked to the presence of R bodies as for killing elicited by kappa.
[e] ND, None detected.
[f] Plants of the Gramineae family (e.g., corn, oats, and barley) are affected, but killing has not been linked unambiguously with the presence of R bodies.

tive animals die within a few hours to several days. Their deaths result from exposure to toxic particles released into their environment by the killers. This is evidenced by simply placing sensitive animals into cell-free medium taken from a killer culture (4, 5). The sensitive animals die even though direct contact between sensitive and killer cells has not occurred.

These findings stimulated considerable interest because Sonneborn was able to demonstrate that toxin production is not controlled by nuclear genes (109). Instead, the genetic determinants for this trait are transmitted according to patterns of cytoplasmic inheritance. Believing that cytoplasmic genes, or "plasmagenes," controlled this trait, and following the conventions of nomenclature at the time, the heritable cytoplasmic factor was named kappa (109).

Kappa particles identified as bacterial endosymbionts. These plasmagenes are now known to reside in symbiotic bacteria living in the cytoplasm of paramecia, and the terms kappa and kappa particle have come to be synonymous with these bacteria. The possibility that kappa particles were bacteria was suggested as early as 1946 (1), and evidence supporting this hypothesis began accumulating shortly thereafter, although some researchers remained unconvinced for many years.

Preer (69), in 1948, reasoned from studies involving X-ray inactivation of toxic particles that kappa particles are about the size of small bacteria. Soon thereafter, Preer (70) was able to visualize kappa particles by light microscopy as Feulgen-positive particles, and he even observed them dividing by binary fission (70). These observations suggested that kappa particles were actually bacterial symbionts of paramecia. This hypothesis was compatible with earlier observations that kappa particles reproduce independently and at a different rate from paramecia themselves (69) and are infective (110) and mutable (22).

In 1958, electron microscopy of sectioned paramecia revealed that kappa particles have the fine structure of procaryotes and cell walls typical of gram-negative bacteria (23, 35). Although the idea of naked cytoplasmic genes had been abandoned by this time, there was still resistance to the idea that kappa particles were bacterial symbionts. According to some, kappa particles were best classified as viruses, while to others they appeared to be rickettsiae. Proposals were also advanced that kappa particles were derived from green algae, or were perhaps "infected" mitochondria (110). Sonneborn (110) stated in 1959 that kappa particles were probably intermediate between bacteria and viruses.

Preer et al. (76) speculated that had kappa particles been discovered first as microscopically visible particles rather than as genetic elements, there would not have been such hesitation to recognize them as symbiotic bacteria. However, the fact that kappa particles cannot be grown outside their hosts undoubtedly added to the confusion over their nature. Considerable efforts have been made toward raising various *Paramecium* endosymbionts outside their hosts, but, at best, growth of the bacteria has been poor. The lambda and mu endosymbionts can be made to grow outside their hosts, but only at about one fission per day, and high-density populations have never been achieved (123, 128). Further questioning of the bacterial nature of kappa particles concerned a lack of biochemical evidence and the absence of a distinct nucleoid region (110). The latter is no longer considered a problem, since the nucleoid region in many bacterial species is difficult to distinguish (76).

Subsequent findings at the biochemical level have now firmly established that kappa particles and other endosymbionts of paramecia are bacteria. The evidence has been summarized by Preer (71) and includes (i) base composition differences between kappa DNA and paramecium DNA (102); (ii) comparable sedimentation values of symbiont ribosomal ribonucleic acid (rRNA) with those of other bacteria (6); (iii) hybridization of symbiont rRNA with *Escherichia coli* DNA but not paramecium DNA (R. Baker, Ph.D. thesis, University of East Anglia, Norwich, England, 1971); (iv) the ability of isolated kappa particles to respire and the presence of enzymes from the glycolytic pathway, pentose phosphate shunt, and citric acid cycle (52); and (v) differences between kappa cytochromes and those of paramecia, but a similarity between kappa cytochromes and those of certain bacteria (52).

Kappa particles associated with killing. Direct contact between killer and sensitive paramecia is not necessary for killing to occur. Culture fluid that has contained killer paramecia for only a few hours is toxic to sensitive paramecia. Austin (4, 5) showed that the toxicity of such fluid is proportional to the number of killers and the length of time they were present in the media. This work led to the conclusion that the toxic agents are discrete particles released from killer paramecia into the environment at a constant rate. It was also noted that one particle is sufficient to kill one sensitive animal. The efforts to identify the toxic particle soon focused on the microscopically visible kappa particles.

Observations of kappa by phase-contrast microscopy showed that kappa populations are subdivided into bright particles (kappa particles possessing a refractile inclusion body, the R body) and nonbright particles (kappa particles without R bodies) (79). The proportion of bright particles in an endosymbiont population typically varies from 1 to 35% but occasionally is as high as 50%, depending upon the particular kappa strain and the nutritional status of the host (a higher percentage of bright particles is seen in starved paramecia) (76, 79).

Following the discovery of bright particles, it was shown that killing activity correlated directly with exposure of sensitive animals to bright particles but was unrelated to exposure to nonbright particles (78). This relationship was confirmed in later studies with purified bright and nonbright particles (66). Thus, isolated bright particles, unlike nonbright particles, are capable of killing sensitive paramecia.

It was also observed that a kappa population can be established within the host paramecium provided that there is at least one nonbright kappa particle to begin with (78). This is not true for bright particles. That is, only nonbright particles are reproductively active and infective (66, 102). This could be demonstrated because, under certain conditions, paramecia lacking kappa particles can incorporate them into their cytoplasm (66, 114, 115). Kappa particles can also be artificially introduced into paramecia by microinjection (27, 51).

Additionally, kappa populations derived from single nonbright particles are found to contain bright particles, indicating that bright particles arise from nonbright particles (78). These particles are also serologically cross-reactive, further demonstrating that they are indeed related (101). Thus, the reproductive form of kappa can transform into a nonreproductive form characterized by the conspicuous presence of an R body and toxigenecity.

Variations in killing activity. Strains of kappa-infected paramecia have been collected from around the world. In all cases, the kappa symbionts confer a killer trait upon their hosts. However, all strains of killer paramecia do not exhibit

the same type of killing. The prelethal symptoms elicited in sensitive paramecia differ among certain strains of killer paramecia depending on the specific type of kappa symbionts that they carry (110).

Prelethal symptoms in affected paramecia are categorized into four basic groups (110). The types of killing are (i) hump killing, in which a large aboral blister develops in affected cells; (ii) spin killing, in which affected cells swim with a direction of rotation opposite to the normal direction of rotation; (iii) vacuolization, in which large vacuoles develop in affected cells; and (iv) paralysis, in which affected cells cease swimming and only occasionally show weak avoidance reactions.

Not only does the particular kappa strain maintained by the paramecium determine the mode of killing by that paramecium, but also the kappa particles provide their host with protection against the toxin that is produced. However, this resistance is specific against a particular group of killers only. Thus, a hump killer strain is resistant to all other hump-killing strains, but is susceptible to spin killers, vacuolizers, or paralyzers. Furthermore, Preer et al. (76) suggested that resistance to specific toxins is not absolute. They pointed to unpublished studies by Preer and Widmayer in which killer paramecia were allowed to grow at different rates so that the kappa densities in these cells also varied. The killers containing fewer kappa particles, although still possessing killing activity against sensitive paramecia, could themselves be killed by exposure to high concentrations of isolated kappa particles of the same strain. This was shown for both spin killers (*C. varicaedens* 7) and hump killers (*C. taeniospiralis* 51).

Killing tests have been conducted against a variety of ciliate species and other organisms. Mueller and Sonneborn (J. A. Mueller and T. M. Sonneborn, Anat. Rec. **134**:613, 1959) reported that killer paramecia had no effect on *Tetrahymena*, *Colpoda*, *Stentor*, or *Cladocera* species or on various species of algae or rotifers. They did, however, find that *Colpidium colpidium* was sensitive to killer paramecia of stock 51. This is the only reported example of an organism other than a paramecium being killed by killer paramecia. It has also been reported (111, 112) that certain stocks of *Didinium nasutum* and *Dileptus* species can become infected with mu, kappa, or sigma bacteria when fed killer paramecia that are hosts to these endosymbionts. These continue to be maintained when the diet is changed to nonkiller paramecia.

Taxonomy of Kappa Particles

Kappa particles make up the genus *Caedibacter*. They are currently divided into five species and are described as gram-negative, nonsporeforming, nonmotile, facultatively anaerobic rods existing as obligate endosymbionts of *P. tetraurelia*, *P. biaurelia*, or *P. caudatum* (76, 85, 95). Four species, *C. taeniospiralis*, *C. varicaedens*, *C. pseudomutans*, and *C. paraconjugatus*, are cytoplasmic endosymbionts in *P. biaurelia* or *P. tetraurelia* (sibling species in the *P. aurelia* species complex). In contrast, the fifth species, *C. caryophila*, discovered in 1978 (25), is a macronuclear symbiont of *P. caudatum*. In further contrast to the other species, which are thought to exhibit mutualistic relationships with their hosts (58), *C. caryophila* has been suggested to be parasitic (25, 94). However, despite differences in microhabitats and host animals, these symbionts possess a singular unifying and conspicuous characteristic: the ability to produce unique refractile inclusion bodies (R bodies) and to confer a killing trait upon their hosts. Their ability to produce R bodies distinguishes them from other *Paramecium* endosymbionts.

In an earlier classification scheme, Preer et al. (76) combined all kappa and pi symbionts (believed to be mutant strains of kappa) into a single species. The original description of the genus *Caedibacter* included kappa and pi symbionts, as well as three other symbionts of paramecia: mu, gamma, and nu. Preer et al. (76) were attempting to organize in a reasonable way the various symbionts carried by paramecia. The available data were nicely accounted for by the construction of three genera (including *Caedibacter*). Support for grouping kappa and pi with mu, gamma, and nu was based primarily upon morphology and killing activities, but also partly on measurements of G+C content and hybridization studies (R. J. Behme, Ph.D. thesis, Indiana University, Bloomington, 1969).

Later, however, independent measurements of G+C content and new hybridization data (20, 84, 85) raised questions concerning how closely related kappa symbionts are to other symbionts. Kappa particles have higher G+C contents than these other bacteria do (40 to 44 mol% versus 35 to 39 mol%) (85). The DNA of all kappa strains tested showed less than 12% homology with that of nonkappa symbionts. Even the supposed kappa mutants, the pi particles, were shown to be quite distinct from kappa (84). Not only were kappa particles distantly related to the nonkappa symbionts, but kappa particles themselves could be separated into four genetically distinct groups. Thus, the superficial similarities and common characteristics shared by *Paramecium* endosymbionts have been shown to be poor indicators of evolutionary relatedness. In light of this information, it would be interesting to use such molecular markers of evolution as 16S rRNA to assess endosymbiont relationships.

The hump killer kappa, which all produce type 51 R bodies, form a coherent group in terms of DNA homologies, but are less than 15% (and in most cases less than 1%) homologous to other kappa strains. Members of a second group of type 7 R-body producers, strains 7, 576, and 310, show high homology with one another (greater than 74%), and moderate homology with strains 1038 and 511 (30 and 45%, respectively). These type 7 R-body producers were consequently viewed as a separate kappa group. The third kappa category consists of a single strain, 51m1. Although 51m1 was previously thought to be a mutant of the hump-killing strain 51, its DNA is less than 6% homologous with DNA from any other kappa strain. Finally, the only R-body producer that exhibits mate killing, strain 570, although not tested, was presumed to be a divergent form of kappa, distinct from other kappa particles and separate from mu symbionts (mu symbionts are mate killers which lack R bodies and are genetically distant from kappa symbionts, having less than 10% DNA homology with kappa symbionts). These four kappa groups, although genetically quite distinct from one another, were brought together as the genus *Caedibacter* for the practical reason that they were all endosymbionts of paramecia and had in common an easily distinguishable and unique characteristic: the ability to synthesize R bodies. The other symbionts, mu, gamma, nu, and pi, were placed into a new genus, *Pseudocaedibacter*, representing three separate species (pi and nu were found to be closely related and so were combined as a single species).

The hybridization data, upon which the Quackenbush classification (85) was largely based, agreed nicely with the categories of kappa described by Preer et al. (80). Thus, kappa species can be differentiated not only by DNA homologies, but also by the following characteristics: R-body

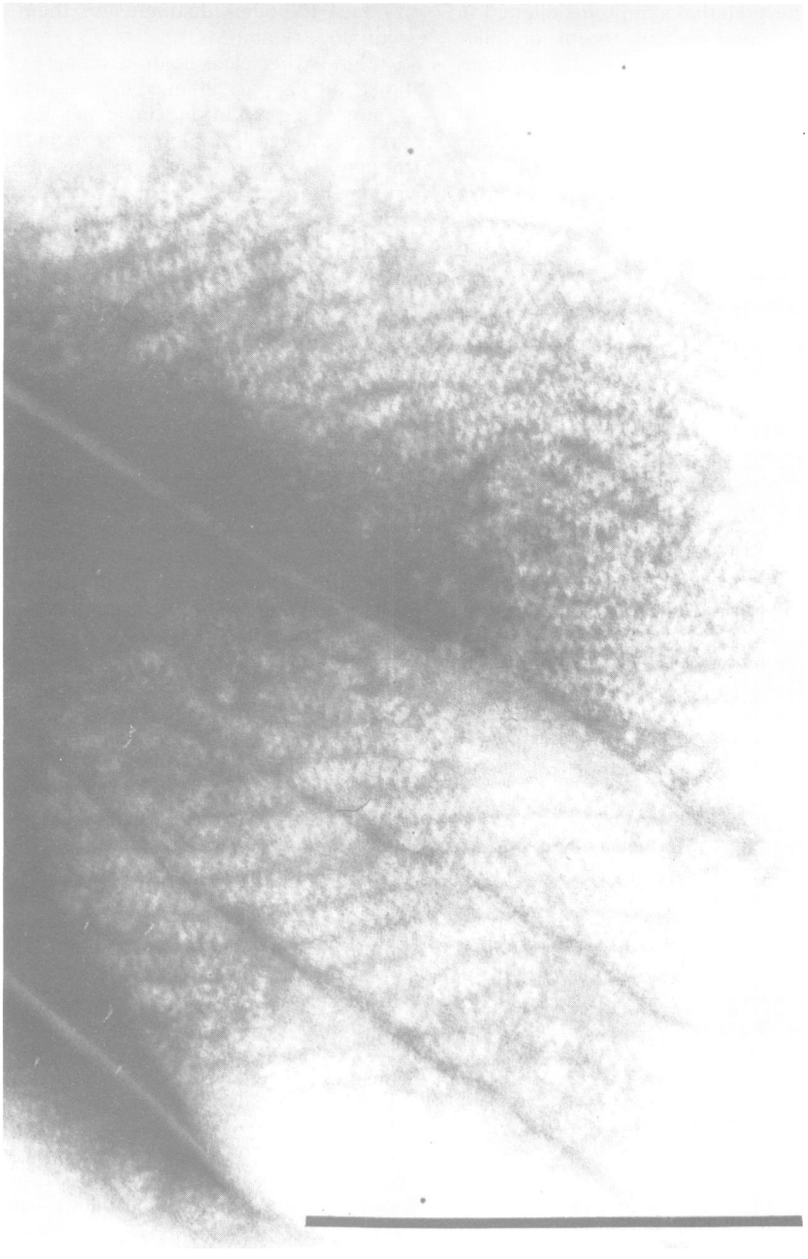


FIG. 2. Helical bacteriophagelike structures in association with an unrolled type 51 R body. Bar, 0.25 μm. Reprinted from reference 80 with permission of the *Journal of Cell Science*.

morphology, associated bacteriophage and bacteriophage-like structures, prelethal symptoms elicited in sensitive paramecia, and host specificities. The following species descriptions emphasize these characteristics. Because of its recent discovery, less is known about the fifth kappa species, *C. caryophila*. A description of this species is included, however, along with the rationale for placing it in the genus *Caedibacter*. Table 2 summarizes the species characteristics.

C. taeniospiralis. Included in this species are strains 47, 51, 116, 169, 298, and A30. These strains all produce type 51 R bodies and are hump killers. In fact, they alone possess these characteristics. Associated with the R bodies are helical structures (Fig. 2) that appear similar to products of aberrant bacteriophage capsid assembly. These kappa particles are symbionts of *P. tetraurelia* and can be purified from their hosts by application of the paramecium homogenate to an epichlorohydrin triethanolamine (ECTEOLA) anion-exchange column; the symbionts pass through the column, whereas host paramecium fragments do not. The cell walls of these kappa particles appear to be more fragile than those of other kappa strains and lyse easily in low concentrations of sodium deoxycholate or when subjected to brief ultrasonication (80).

Although most *C. taeniospiralis* strains share substantial DNA homology (greater than 80%), strains A30 and 51 share

only 41% DNA homology (85). Nevertheless, when compared with other kappa species, the hump killers are quite distinct. In all *C. taeniospiralis* strains, the genetic determinants for R-body production are plasmid borne, and the plasmids isolated from the different strains share a high degree of homology. In other kappa species, plasmids have not been detected, and R bodies are probably encoded by bacteriophages (80, 90).

C. varicaedens. Quackenbush et al. (85, 90) proposes that *C. varicaedens* be divided into three subspecies. This is reasonable in terms of both DNA homologies (see above) and certain well-defined distinguishing characteristics. These kappa particles are symbionts of *P. biaurelia*. All members of this species produce type 7 R bodies, but are associated with different types of killing activities.

C. varicaedens subspecies 7 includes strains 7, 310, 576, and 1039. They differ in the way they kill sensitive paramecia. Spin killing, vacuolization, and paralysis are all represented. Icosahedral bacteriophages are associated with the R bodies (Fig. 3). Capsomerelike structures (about 10 nm in diameter) are also seen in these strains. The R bodies are said to be sticky; that is, bacteriophages and capsomeres attach to the inner end of the R-body ribbon (Fig. 4). Occasionally, in strain 576, bacteriophages are seen with tails. The strains belonging to this subspecies are also notable because a membranous sheath surrounds the R bodies and the isolated R bodies exhibit killing activity. These kappa particles irreversibly bind to ECTEOLA but can be purified from their hosts by passage of the paramecium homogenate through a filter paper column; the kappa particles, but not the paramecium fragments, are retained on the column and then eluted when the ionic strength and pH of the column buffer are increased.

C. varicaedens subspecies 562 includes strains 511, 517, and 562. These kappa particles all kill by vacuolization. Icosahedral bacteriophages are associated with R-body synthesis, but are not attached to the R body (Fig. 5). There is no sheath around the R bodies, and no capsomerelike structures have been observed. These kappa particles do not adsorb to either ECTEOLA or filter paper and so are purified in the same way that *C. taeniospiralis* strains are. DNA-DNA hybridization reveals only 45% homology between strains 511 and 7.

C. varicaedens subspecies 1038 is represented only by strain 1038. This kappa strain shares many characteristics with the subspecies 7 strains. It is a spin killer, the R bodies are sticky and possess a sheath, and capsomerelike structures are present. The strain is also purified in the same way as the subspecies 7 strains are. The difference between these subspecies is that strain 1038 possesses helical structures similar to those of *C. taeniospiralis* (Fig. 6) and the R bodies show little, if any, killing activity. DNA-DNA hybridization reveals only 38% homology between strain 1038 and strain 7.

C. pseudomutans. *C. pseudomutans* is represented only by strain 51m1. Although it was originally thought to be a mutant of *C. taeniospiralis* 51, this has been shown not to be true (see above). This strain produces type 7 R bodies (which are sticky), icosahedral bacteriophages, and capsomerelike structures. No killing activity has been noted with isolated R bodies. These kappa particles are spin killers and are purified in the same way as the subspecies 7 kappa particles are. Thus, this kappa species is superficially quite similar to *C. varicaedens*. However, *C. pseudomutans* strains are symbionts of *P. tetraurelia* and are genetically distinct from all other kappa species (less than 6% DNA homology with other strains).

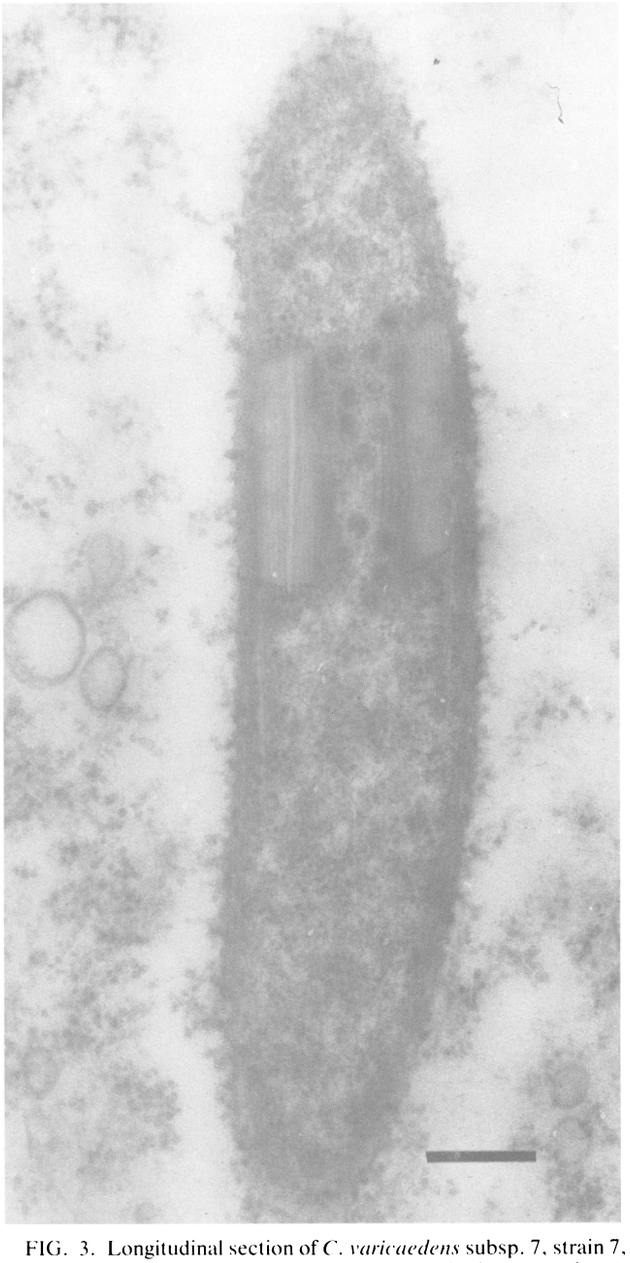


FIG. 3. Longitudinal section of *C. varicaedens* subsp. 7, strain 7, showing bacteriophages inside a coiled type 7 R body. A membrane sheath is seen surrounding the R body and extending beyond it on either side. Bar, 0.2 μm. Reprinted from reference 73 with permission of *Genetical Research*.

C. paraconjugatus. *C. paraconjugatus* is represented by a single strain, 570, and is very different from the other kappa species. The R bodies are type 7 but are considerably smaller than those of other strains (0.2 μm wide and 5 μm long in comparison with 0.5 μm wide and 10 to 15 μm long). Also, bright kappa particles in this strain are always found at low frequency (about 1 to 2%). The R bodies are sticky and are associated with spherical bacteriophages. No sheath or capsomerelike structures are seen, nor is killing activity associated with the isolated R bodies. This species is further differentiated because the hosts are mate killers. Isolated

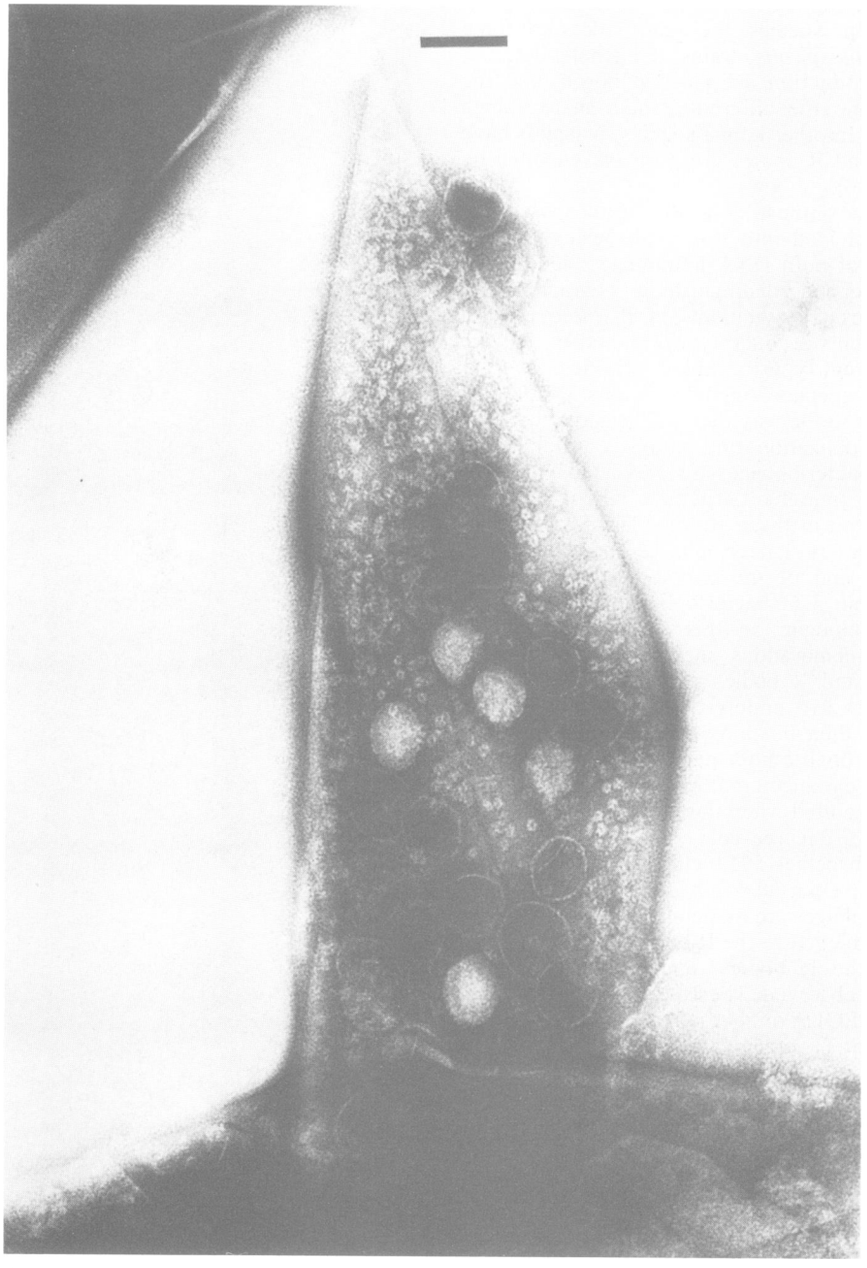


FIG. 4. Inside tip of an unrolled type 7 R body from *C. varicaedens* subsp. 7, strain 7, showing bacteriophages and capsomerelike structures. Bar, 0.1 μm. Reprinted from reference 74 with the permission of the authors.

whole brights do not exhibit killing. Killing occurs when their host paramecium comes into physical contact with a sensitive paramecium, such as during conjugation. However, Gibson (28) reported that toxin can be transmitted at times other than during conjugation.

Agglutination of cells for only 1 hour, without subsequent fusion and completion of the conjugation process, is sufficient for killing to take place (30). Death of the sensitive cell is characterized by a shrinking or shriveling. This type of killing has been reported for paramecia that maintain the non-R-body-producing mu symbionts, *Pseudocaedibacter conjugatus* (28). The mate-killer kappa particles are symbionts of *P. biaurelia*.

C. caryophila. R-body-containing macronuclear endosymbionts were first observed in a *P. caudatum* strain collected in northern France near Amiens (25). Schmidt et al. (94–96) recently found a similar macronuclear R-body-containing endosymbiont in a *P. caudatum* strain collected in Münster, Federal Republic of Germany (Fig. 7). It is not yet known to what extent the kappa strains from France and the Federal Republic of Germany are similar. The symbiont isolated from the Federal Republic of Germany has been shown to possess features that warrant its inclusion in the genus *Caedibacter*, and it has been given the name *C. caryophila* (95). It is a *Paramecium* symbiont, mediates the expression of a killer trait in its host, and produces R bodies. Therefore,

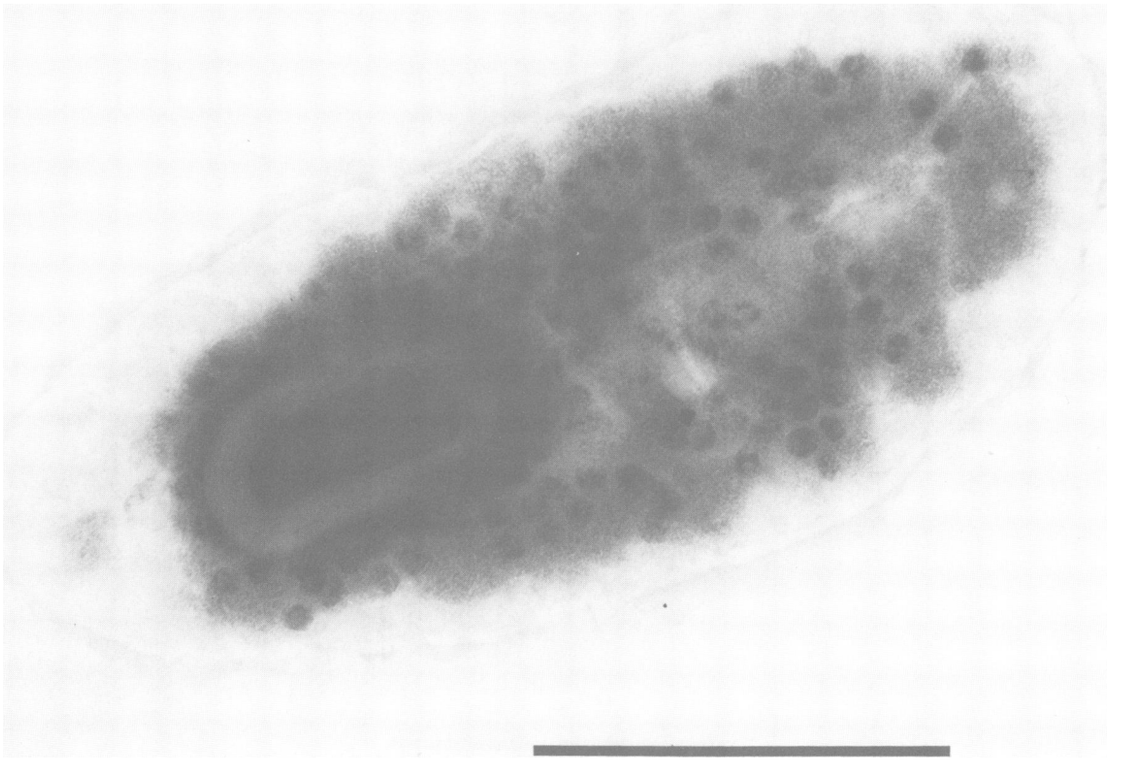


FIG. 5. Section of *C. varicaedens* subsp. 562, strain 562, showing bacteriophages outside the coiled type 7 R body. Bar, 1.0 μm. Reprinted from reference 80 with permission of the *Journal of Cell Science*.

these macronuclear endosymbionts are considered to represent a new species of kappa.

The R bodies of *C. caryophila* are larger than the other kappa R bodies (96). Cc R bodies unroll like type 51 R bodies (Fig. 8) but their ends resemble those of type 7 R bodies (Fig. 9). Associated with the R bodies are spherical bacteriophages instead of the helical structures seen in *C. taeniospiralis* (Fig. 10). The macronuclear kappa particles resemble *C. varicaedens* further in having a cigar-shaped appearance rather than the more oval appearance of *C. taeniospiralis*.

Nature of Kappa-*Paramecium* Symbioses

Preer et al. (76), in their 1974 review, described kappa particles simply as endosymbionts. The behavior of kappa-bearing paramecia in the laboratory suggested to them that the relationship between kappa particles and their hosts was mutualistic, since kappa particles confer upon paramecia the ability to kill and generally do not harm their hosts, and they certainly benefit from the relationship. However, Preer et al. (76) preferred to describe the relationship simply as a symbiosis, since it was uncertain what effects kappa particles have upon paramecia in nature. Since that time, kappa particles have generally been portrayed as maintaining a mutualistic relationship with their hosts, although one kappa species has been labeled parasitic.

Estevè found that *P. caudatum* populations infected with *C. caryophila* and cultured under starvation conditions did not survive beyond 25 days, whereas kappa-free cultures kept under identical conditions were not killed (25). He concluded that these symbionts were actually parasitic. Furthermore, Schmidt et al. (94) have studied two strains of macronuclear kappa particles in *P. caudatum*, one of which has spontaneously lost the ability to produce R bodies. They observed that only the R-body-producing strain harmed the host under starvation conditions similar to those described by Esteve (25). However, it is considered that other kappa species maintain mutualistic relationships with their hosts (58).

Preer et al. (76, 82) postulated that since infection by kappa particles is apparently a rare occurrence, kappa-bearing paramecia probably persist in natural environments because they have a selective advantage over sensitive paramecia, at least under certain conditions. Landis (58) confirmed that in mixed cultures, kappa-bearing paramecia are selected over kappa-free paramecia, presumably owing to the toxic traits associated with kappa.

However, there remain questions concerning the validity of extrapolating from laboratory studies to nature. For example, the negative consequences of harboring the "parasitic" *C. caryophila* may be negligible under conditions that favor paramecium growth and may be offset in nature by the selective advantage gained by being a killer. Presumably, *P. caudatum* obtains some benefit from these symbionts, since *C. caryophila* confers upon its host the paralysis-killing trait. Certainly, kappa particles derive their nutrients from their hosts, but to what extent this constitutes a metabolic burden to the paramecia is uncertain. It has been shown that the

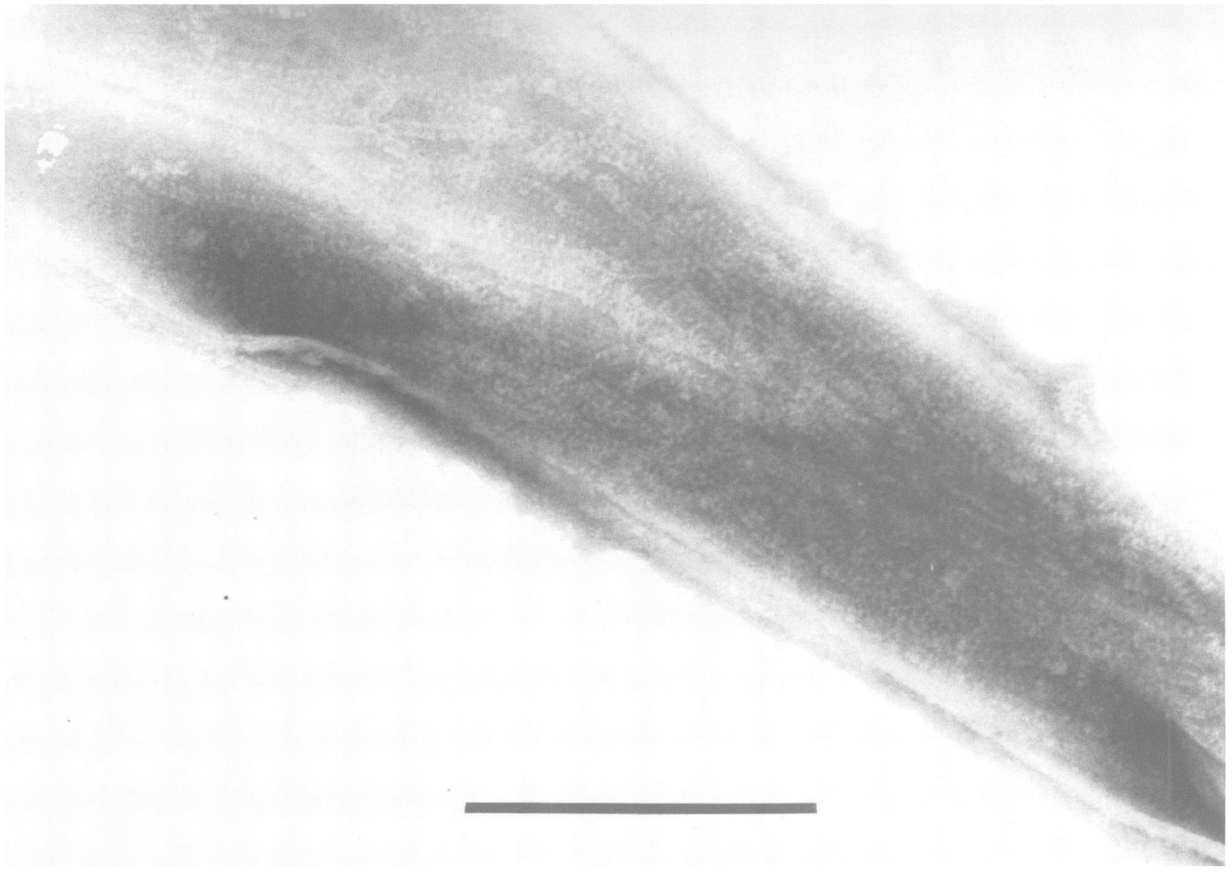


FIG. 6. Helical bacteriophagelike structures in association with an unrolled type 7 R body from *C. varicaedens* subsp. 1038. Bar, 0.25 μm. Reprinted from reference 80 with permission of the *Journal of Cell Science*.

balance between paramecia and cytoplasmic kappa particles is perturbed when the paramecia are grown in certain axenic media, resulting in death of paramecia via rapid symbiont growth (107; R. L. Quackenbush, personal communication). Whether this is significant in nature is doubtful. Different culture conditions allow the paramecia to flourish to the detriment of the symbionts. Apparently a delicate balance exists between host and symbiont, such that there is a fine line between conditions under which the relationship appears mutualistic and those under which it appears parasitic. Although the benefits of the kappa-*Paramecium* relationship to the kappa particles are obvious and toxigenecity is assumed to give paramecia a competitive edge in nature, the labels parasitism and mutualism perhaps should be used only in reference to a particular environmental context.

In other endosymbiont-ciliate relationships, the label of mutualism applies more clearly. Lambda strain 299 has been demonstrated to provide folic acid to its host paramecium (103, 105, 106). The relationship between the symbiont omikron and its host *Euplotes aediculatus* is quite dramatic in that each organism is dependent upon the other for survival (38). Omikron is an obligate symbiont of *Euplotes aediculatus*. Heckmann (38) has demonstrated that the host ciliate is unable to reproduce when large numbers of its symbionts are lost by irradiation or treatment with penicillin. Heckmann also showed that the host cells can recover and become reproductively active when omikron symbionts are reestablished in the cell. Although this type of obligate relationship between symbiont and host appears unique to omikron and *Euplotes* species, it is also plausible, as suggested by Soldo (103), that paramecia derive benefits from their endosymbionts that we have been unable to detect. Certainly, there remains much that is not understood about the establishment and maintenance of symbiotic relationships between bacteria and ciliates.

Finally, in considering the nature of kappa-*Paramecium* symbiosis, we must remember that the involvement of kappa particles in this relationship is obligatory and that only certain paramecia are capable of maintaining kappa symbionts. In *P. biaurelia* and *P. tetraurelia*, maintenance genes are needed for kappa particles to exist in the cytoplasm (109). That is, only certain genotypes allow symbiosis with kappa particles. K is the allele allowing kappa particles to be maintained, and k is an allele that does not allow their maintenance (109). The alleles are apparently codominant, since paramecia that are heterozygous maintain only about half the usual number of kappa particles found in paramecia having the KK genotype (7, 14, 15). Whether *C. caryophila* infects only certain genotypes of *P. caudatum* is not known.

Other *Paramecium* symbionts are also dependent upon maintenance genes and therefore infect only certain *Paramecium* strains. Furthermore, the required maintenance genes are specific for certain symbionts. For example, *P. tetraurelia* strains maintain kappa but not alpha (126). This specificity of maintenance systems does not appear to extend to different kappa species, since microinjection studies

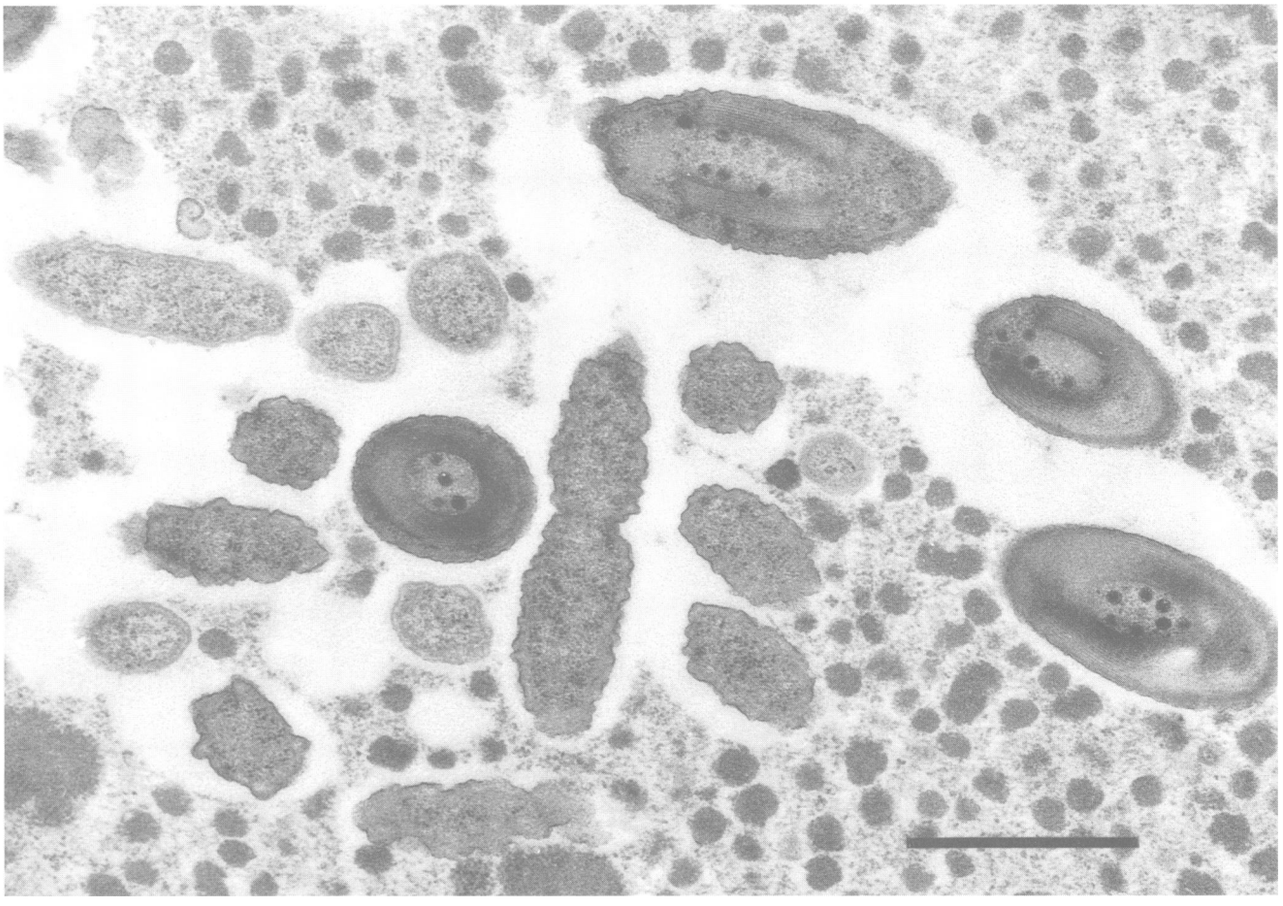


FIG. 7. Electron micrograph of *C. caryophila* 221 in the macronucleus of *P. caudatum*, showing bacteriophages inside coiled type Cc R bodies. Bar, 1.0 μm. Micrograph courtesy of H. Schmidt.

have shown that *C. varicaedens* from *P. biaurelia* can successfully establish populations in *P. tetraurelia*, normally the host for *C. taeniospiralis* and *C. pseudomutans* (28, 51).

It is not known how maintenance genes affect endosymbionts. Chao (14, 15) found that kappa reproduction and, consequently, the kappa density in the host paramecium varied with the paramecium life cycle. Possibly the maintenance genes are expressed differently during the life cycle of the cell and affect kappa reproduction (28). The topic of maintenance genes has been dealt with more extensively in earlier reviews (28, 76), and the reader is referred to these papers for additional details.

Kappa R Bodies

R bodies are diagnostic structures, identifying *Paramecium* endosymbionts as kappa particles. Furthermore, structural and behavioral differences define three classes of kappa R bodies: type 51, type 7, and type Cc (named after the strains in which they were first found). Each kappa species produces one type of R body. Only *C. taeniospiralis* produces type 51 R bodies, and only *C. caryophila* produces type Cc R bodies. Other kappa species produce type 7 R bodies. Type 51, type 7, and type Cc R bodies are found exclusively in obligate endosymbionts of paramecia.

R bodies are highly insoluble protein ribbons (3, 74, 77). Types 51 and 7 have similar dimensions (approximately 13 nm thick, 0.5 μm wide, and 10 to 15 μm long) (3). They are normally seen as tightly rolled cylindrical structures, about 0.5 μm in diameter, with a small opening (0.25 μm in diameter) through the center. Both types of R bodies can, however, unroll.

When unrolled, the type 51 R-body ribbon is seen terminating in an acute angle at each end. This is not the case for type 7 R bodies. Although the inner end of the rolled type 7 R body forms an acute angle, the outer end is blunt or irregular (72) (Fig. 11). Another difference between these classes of R bodies is the way unrolling occurs. Unwinding of type 7 R bodies occurs from the outside (Fig. 12) and is irreversible. Also, type 7 R bodies are not responsive to pH changes, but instead can be triggered to unroll by being heated to 70°C or treated with sodium dodecyl sulfate (SDS) or phosphotungstate (72). Type 51 R bodies unwind, at pH values less than 6.5, in a telescoping fashion from the inside, and they reroll when the pH increases above 7.0 (72) (Fig. 13 to 15). However, whether unrolling and rerolling are strictly due to changes in pH has been questioned, since Bedingfield et al. (9) reported that concentrations of particular ions may induce unrolling. They found that ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA) and ethylenediaminetetraacetic acid (EDTA) at 0.1 M and pH 8.5 cause type 51 R bodies to unroll. Additional work remains to determine whether calcium or other ions are important in the

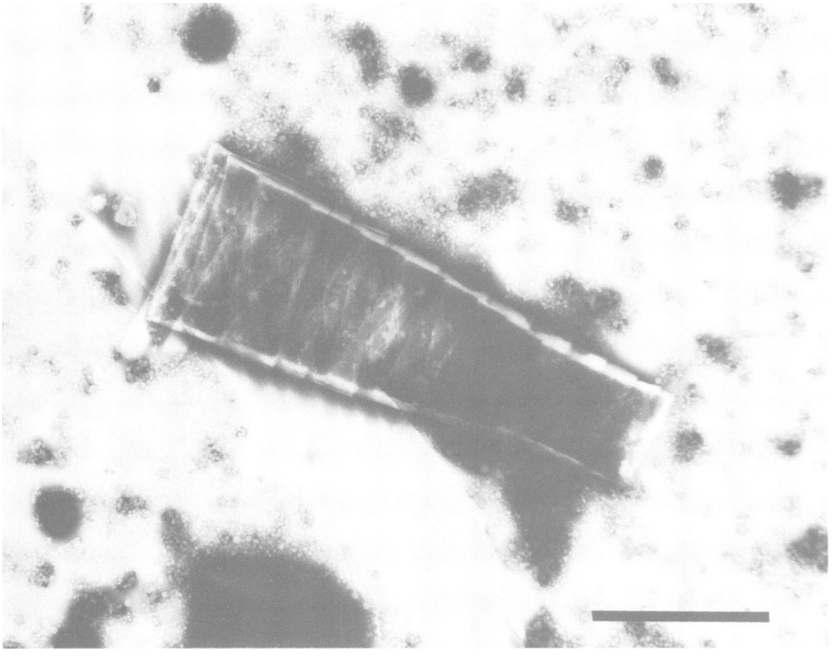


FIG. 8. Type Cc R body beginning to unroll from inside in a telescopic fashion. Bar, 1.0 μm. Micrograph courtesy of H. Schmidt.

coiling of type 51 R bodies. Gibson et al. (31) confirmed these findings and also showed that urea concentrations greater than 0.5 M promote unrolling of type 51 R bodies. They also reported that type 51 R bodies can be induced to unroll by heating (even at temperatures as low as 30°C, 100% of R bodies were unrolled).

Type Cc R bodies have not been studied to the extent that the other kappa R bodies have, but they are clearly different from both type 51 and type 7 R bodies. Like type 51 R bodies, type Cc R bodies unroll from the inside, in a telescopic fashion (Fig. 8), although it has yet to be determined what stimuli trigger the unrolling process. However, like type 7 R bodies, only the inner terminus of the type Cc R-body ribbon is sharply tapered; the outer end is nearly blunt (Fig. 9). Type Cc R bodies are larger than type 51 and type 7 R bodies: 0.8 μm in diameter and thickness versus 0.5 μm.

R bodies are resistant to treatments that normally denature or solubilize proteins. Preer and Preer (74) were able to solubilize type 7 R bodies only in 70% formic acid. In recent studies (48), it was concluded that covalent linkages other than disulfide bridges were involved in the polymerization of

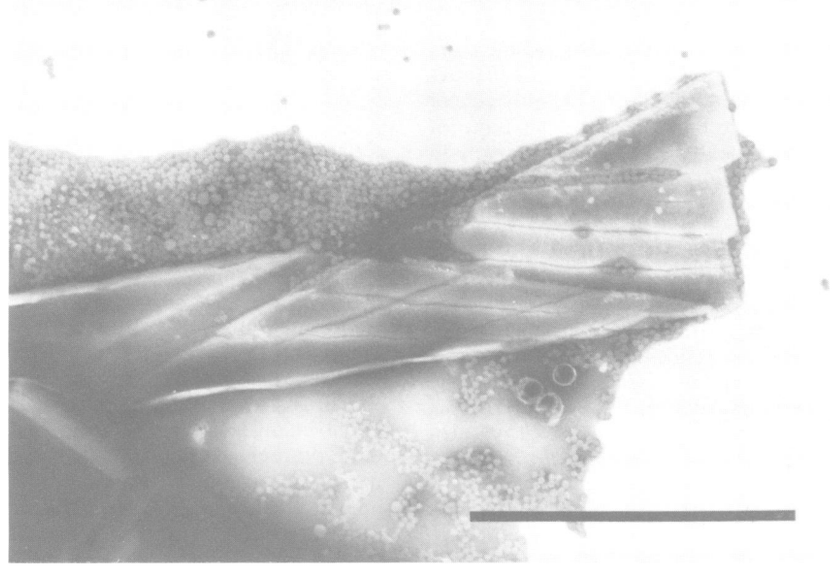


FIG. 9. Outer end of an unrolled type Cc R body. Bar, 1.0 μm. Reprinted from reference 96 with permission of the *Journal of Cell Science*.

FIG. 10. Inner tip of an unrolled type Cc R body showing associated bacteriophages. Bar, 0.5 μm. Micrograph courtesy of H. Schmidt.

type 51 R bodies since these R bodies are resistant to treatments that would otherwise dissociate these structures. Less is known about the assembly of type 7 and type Cc R bodies, but they do show resistance to denaturing treatments.

Extrachromosomal Elements

The synthesis of R bodies was proposed as early as 1959 (110) to be linked to prophage induction, and, indeed, this relationship appears to exist for organisms that produce type 7 and type Cc R bodies. In contrast, synthesis of type 51 R bodies has been clearly demonstrated to be determined by plasmid DNA (89). That different extrachromosomal elements are involved in R-body synthesis is particularly interesting since these R bodies probably have a common evolutionary origin. The presence of R-body-encoding extrachromosomal elements in kappa species may provide an explanation for a common origin of kappa R bodies even though kappa species are not themselves closely related.

Bacteriophages. The hypothesis that R-body production is directed by bacteriophages was first inferred from the observations that (i) the transformation of nonbright into bright particles involves the simultaneous expression of R bodies and toxin, and (ii) kappa particles that contain R bodies are not themselves reproductively active, but rather arise from the reproductively active nonbright kappa particles (110). It was proposed that nonbright kappa particles might be lysogenic, with prophage induction resulting in loss of reproductive ability and expression of bright characteristics (i.e., R-body production and killing activity).

Subsequent findings confirmed this hypothesis for type 7 R bodies. Electron microscopy revealed an association of icosahedral bacteriophagelike structures with type 7 R bodies (34, 73, 74). These icosahedral structures were observed only in bright particles. Also seen in some kappa strains are capsomerelike structures and apparent tail structures (80). In rare instances, in strains 576 and 562, bacteriophages with tails are seen. The capsomerelike structures may be unassembled bacteriophage elements, possibly tail subunits (80), although there are no data to substantiate this.

Preer et al. (77) isolated bacteriophages from *C. varicaedens* 562 and showed that DNA was packaged in these structures in amounts comparable to those in known bacteriophages (about 16 fg of DNA, or 80% of the DNA content of bacteriophage T2). Furthermore, this DNA had a density significantly lower than that of chromosome DNA ($\rho = 1.700$ g/cm^3 for bacteriophage DNA and 1.702 for whole kappa DNA). These findings led to the conclusion and general acceptance that kappa particles do contain bacteriophages.

Quackenbush et al. (90) proposed a system for naming these bacteriophages. Their system is used in this paper. Each bacteriophage is given a two- or three-letter prefix corresponding to the initials of the species name of the host kappa particle. This prefix is followed by the strain number of the host kappa particle. For example, the bacteriophages found in *C. varicaedens* 7 are named Cv7, and bacteriophage Cps51m1 is found in *C. pseudomutans* 51m1.

Quackenbush (R. L. Quackenbush, Ph.D. thesis, Indiana University, Bloomington, 1977) has shown by hybridization kinetics that the bacteriophage Cv562 genome is smaller than the host genome. Also, the bacteriophage genome is amplified with respect to the chromosomal DNA. The sequence complexity of chromosomal DNA compares favorably with that of bacteria. The sequence complexity of DNA isolated from bacteriophages measures only 40×10^6 daltons, indicating that the bacteriophages are not packaging chromosomal DNA.

Amplified DNA sequences were also found in *C. pseudomutans* and other *C. varicaedens* strains and presumably represent amplification of bacteriophage genomes (90). The correlation between the appearance of bacteriophage structures and the occurrence of R bodies and toxigenecity suggest that bright characteristics are indeed bacteriophage encoded.

There is also evidence that the R bodies found in *C. caryophila* are encoded by a bacteriophage. Two strains of *C. caryophila* are known (94, 95): 220 and 221. Strain 220 is quite interesting because it has apparently lost the ability to produce R bodies and exhibits no killing activity. Strain 220 was discovered in a *P. caudatum* strain collected from a pond in Münster, Federal Republic of Germany. Populations of this kappa strain possessed R-body-containing bright particles. Symbiont-bearing paramecia were maintained in the laboratory for about 3 months, at which time it was noted that bright particles were no longer present in symbiont populations (94). Paramecia that contained R-body-producing symbionts were again collected from the pond. The second strain, termed 221, has been maintained for nearly 3 years in laboratory cultures, and bright kappa particles are still present in the macronuclei. Kappa strains 220 and 221 are believed to be related because DNA from *C. caryophila* 220 and the nonbright forms of *C. caryophila* 221 gave identical patterns when the fragments obtained after restriction endonuclease digestion were separated by agar-

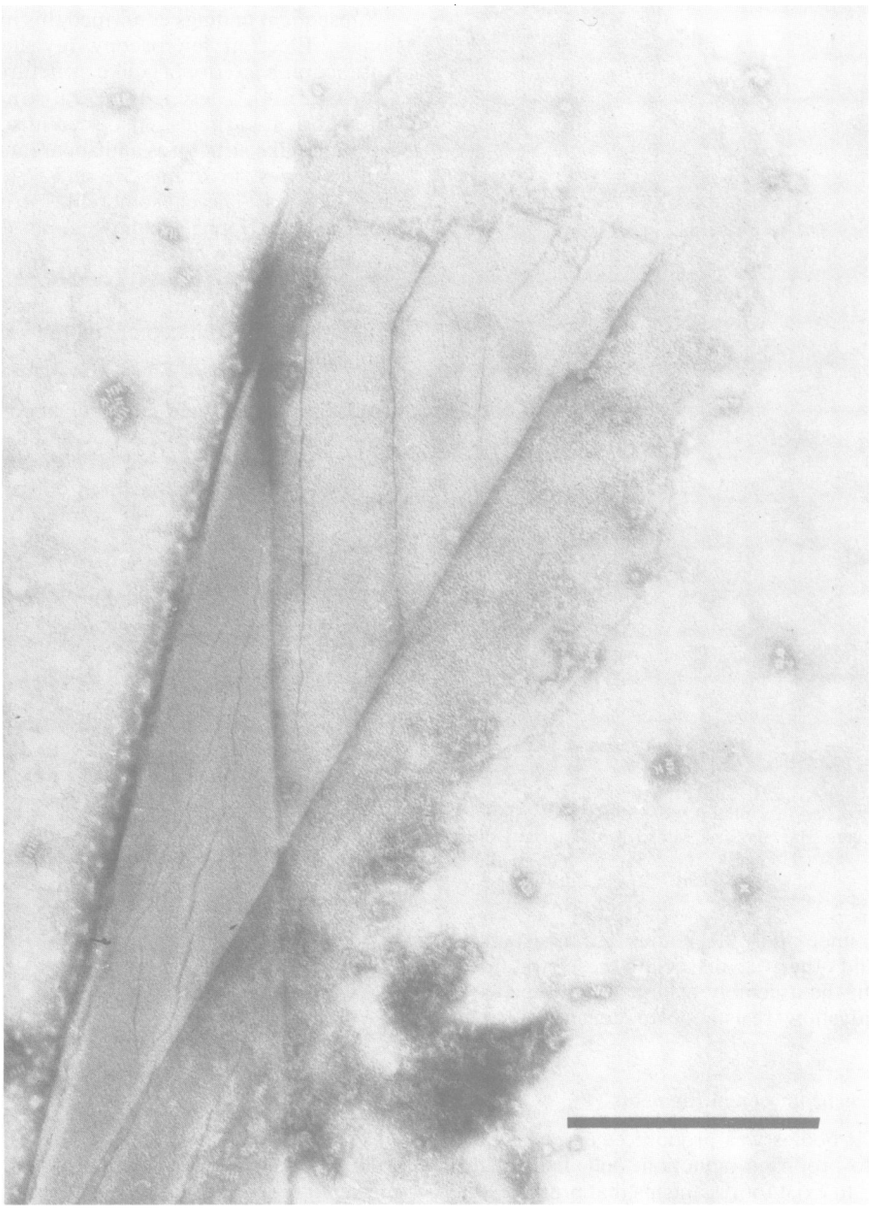


FIG. 11. Outer end of an unrolled type 7 R body from *C. varicaedens* subsp. 7, strain 7. Bar, 0.2 μm. Reprinted from reference 73 with permission of *Genetical Research*.

ose gel electrophoresis (94). The DNA from bright forms of *C. caryophila* 221 gave bands that are different from both the nonbright particles of this strain and *C. caryophila* 220. Since *C. caryophila* is known to possess bacteriophages, and since bacteriophage genomes are known to be amplified in the bright forms of other kappa species, it is likely that bacteriophage DNA has been amplified in the bright forms of *C. caryophila* 221 and that the fragments generated by restriction endonuclease digestion of this DNA are masking the banding patterns of *C. caryophila* 221 chromosomal DNA. Also, DNA from strain 221 hybridizes with Southern blots of DNA from strain 220 (94). These findings suggest that *C. caryophila* R bodies are encoded by the bacteriophage genome, and elimination of R-body production in strain 220 is possibly due to inefficient replication of the bacteriophage genome, reminiscent of the 51m43 mutant of *C. taeniospiralis* (127).

In contrast to all other kappa strains, *C. taeniospiralis* strains and *C. varicaedens* 1038 do not possess icosahedral structures, but they do contain smaller helical structures (about 18 nm wide and of indeterminant length) (80). These helical structures occur exclusively in bright particles, that is, in association with R bodies, as do the spherical bacteriophages of other strains. Although these structures were originally thought to be viral particles, it was later suggested that they represent incomplete or abortive assemblies of bacteriophage components (76). The relation of these structures to the icosahedral bacteriophages seen in other kappa

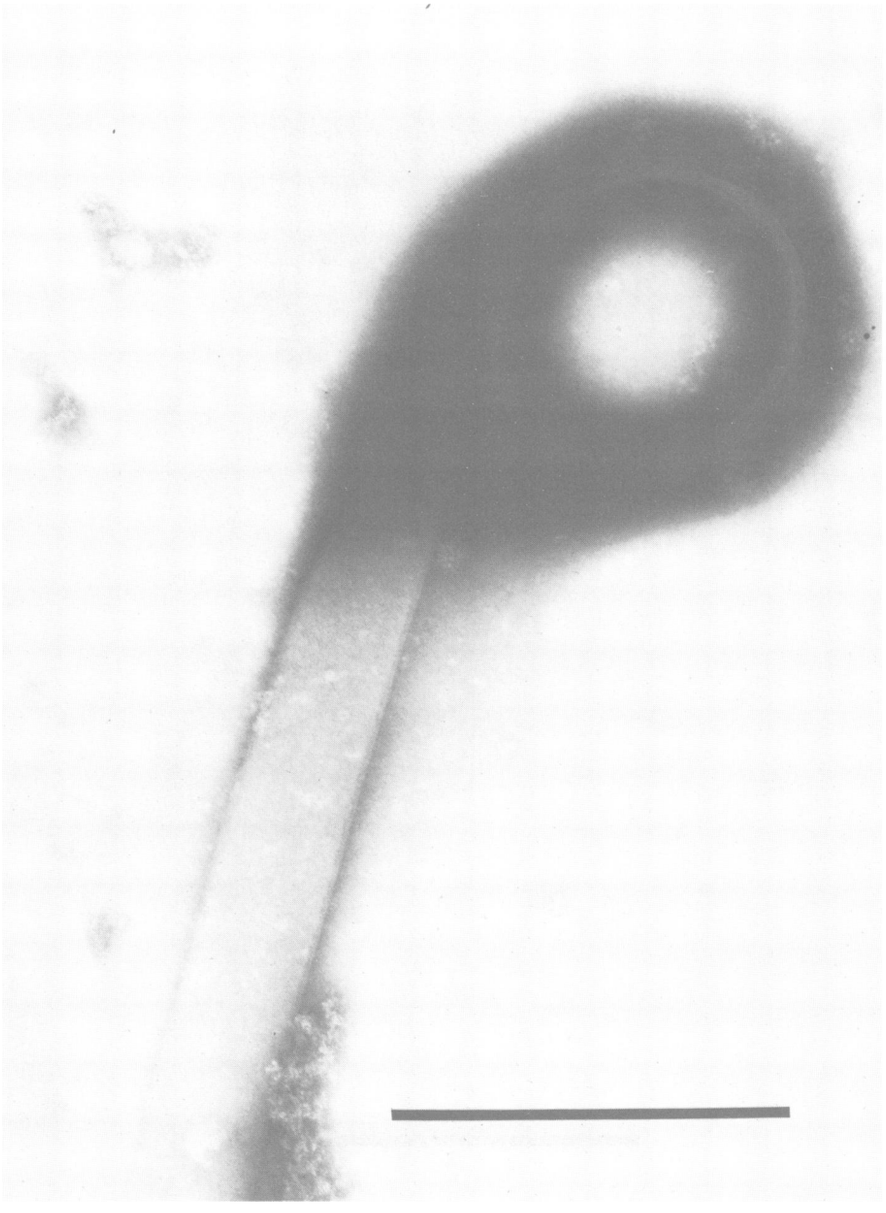


FIG. 12. Type 7 R body from *C. pseudomutans* 51m1 unrolling from the outside. Bar, 0.5 μm. Reprinted from reference 80 with permission of the *Journal of Cell Science*.

particles remains undetermined. However, the presence of bacteriophages has been suggested on the basis that, on exposure to ultraviolet light (a treatment known to induce certain prophages), there is a concomitant increase in the percentage of bright particles in *C. taeniospiralis* 51 populations, that is, an induction of the synthesis of helical structures and R bodies (83). This observation has not been pursued further but is intriguing since it is now known that type 51 R bodies are plasmid encoded (89).

Although bacteriophages apparently direct the synthesis of type 7 R bodies and toxin, they are evidently noninfective, and no evidence exists that bacterial lysis occurs. However, various degrees of structural and functional completeness have been noted for kappa bacteriophages. Preer et al. (80) noted that in one strain, *C. varicaedens* 1039, few spherical headlike structures were observed and they were nearly always found empty. Bacteriophages in *C. varicaedens* 249, 562, 576, 1039, and 1041 are occasionally seen with attached tails (80). Hump-killing kappa particles (*C. taeniospiralis*) become fragile once R-body production has been induced (80), and the bright forms of the macronuclear kappa particles (*C. caryophila*) are noticeably more fragile than those of the hump killers (94, 96). Although lysis of kappa particles has not been reported, is it possible that increased fragility seen in bright forms of these kappa strains represents vestiges of lytic activity? The bacteriophages found in kappa particles are certainly defective, but perhaps to different degrees.

Plasmids. *C. taeniospiralis* is the only kappa species producing type 51 R bodies and also the only species harboring plasmids. Dilts (19) found that *C. taeniospiralis* 51 possesses plasmid DNA (approximately 28 megadaltons

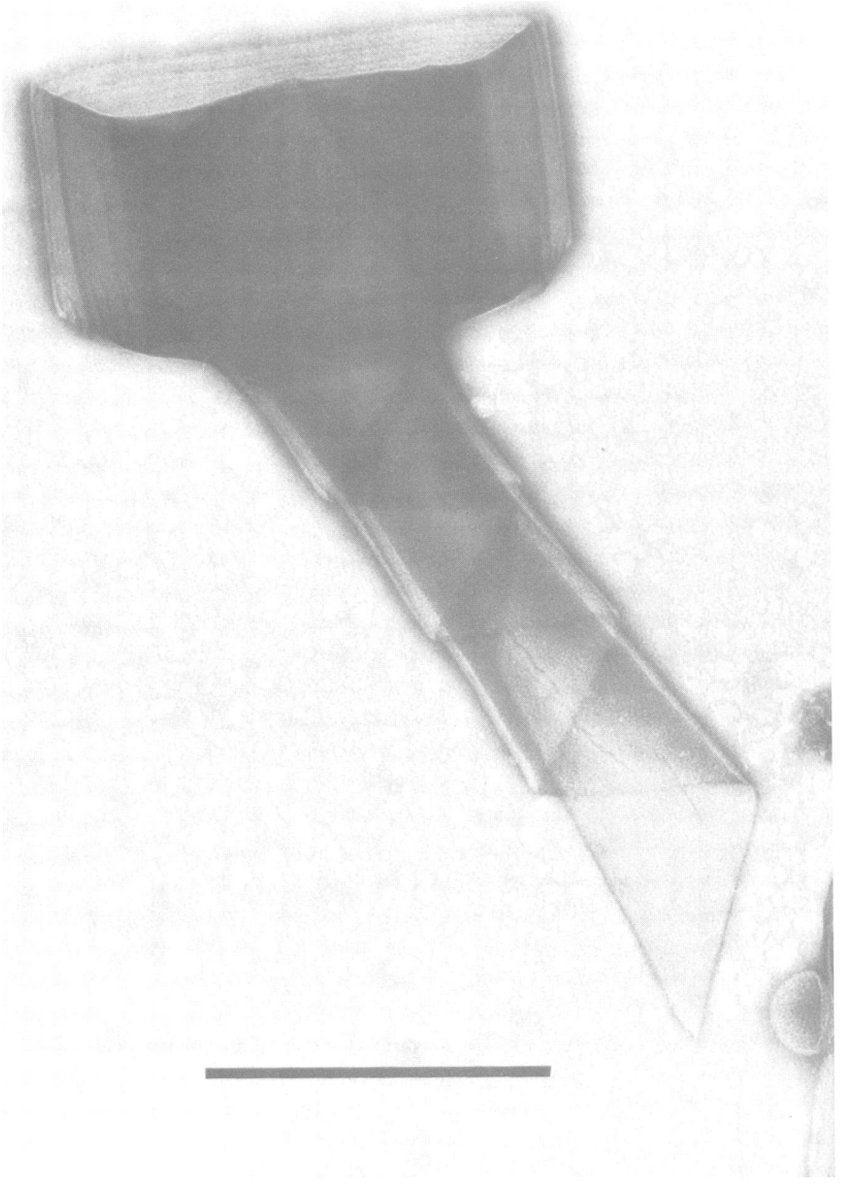


FIG. 13. Type 51 R body unrolling from the inside in a telescopic fashion. Bar, 0.5 μm. Reprinted from reference 74 with permission of the authors.

[MDa]) that exhibits amplified copy number in bright particles. She also determined that the density of plasmid DNA differs from that of chromosome DNA (ρ = 1.698 g/cm^3 for the plasmid DNA and 1.700 g/cm^3 for chromosome DNA). Thus, in this species, plasmid DNA appears to be associated with bright characteristics (R bodies and toxigenicity) in the manner that bacteriophage DNA is associated with these characteristics in other kappa species.

In later studies with the same kappa strain, a larger plasmid (94 MDa) was discovered (20, 92). Hybridizations showed that this plasmid was not related to the previously described plasmid (91). This larger plasmid species constitutes only a small percentage of total plasmid DNA, apparently not being amplified as is the 28-MDa plasmid when R-body synthesis is induced.

Further investigations revealed that plasmids are present in all strains of *C. taeniospiralis* (Fig. 16). In addition, the plasmids of different strains are highly homologous, as determined by restriction endonuclease mapping (86) and DNA-DNA hybridizations (91). Plasmid sizes vary among kappa strains, ranging from 41.5 to 49 kilobase pairs (kbp). These size differences can be accounted for by the presence of 1.5- and/or 7.5-kbp DNA sequences inserted at various sites in the plasmids. These sequences have been identified as transposons (21, 90, 91). R-body-coding plasmids have been assigned the prefix pKAP, which is followed by the

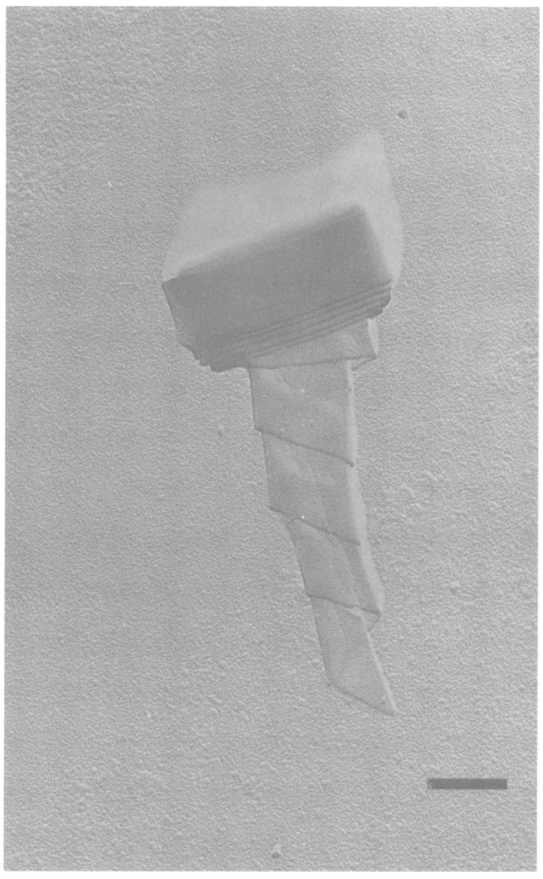


FIG. 14. Type 51 R body unrolling from the inside in a telescopic fashion. Bar, 0.5 μm. Micrograph courtesy of B. Wells.

kappa strain number in which the plasmid in question naturally occurs. Thus, the R-body-encoding plasmid found in *C. taeniospiralis* 47 is designated pKAP47.

About 70% of pKAP47, the R-body-encoding plasmid isolated from *C. taeniospiralis* 47 (89), and about 98% of pKAP116, the R-body-encoding plasmid from *C. taeniospiralis* 116 (F. R. Pond, unpublished results; D. P. Heruth, Ph.D. thesis, University of South Dakota, Vermillion, 1987), have been cloned into *Escherichia coli*. The R-body-encoding regions of these plasmids have been isolated, and R-body production has been expressed in *E. coli* (48, 89). Thus, it has been established that plasmids direct the synthesis of type 51 R bodies, and, although not yet demonstrated, it is believed that toxigenicity is also plasmid encoded in this species.

Transposons. When plasmids from the various *C. taeniospiralis* strains were mapped by restriction endonuclease analysis (86), it was noted that they showed remarkable homology. Each plasmid contains one or two inserted sequences (1.5 or 7.5 kbp) which, if eliminated, would result in identical restriction maps for every plasmid (Fig. 16). These inserted sequences are located at various positions in the plasmids. The insertion sequences have been identified as transposons (21, 90, 91). Restriction maps and hybridization data indicate that there are three different transposons present in these plasmids but that all the transposons are related (91). There are two related 1.5-kbp transposons (Tn*4501* and Tn*4502*) and a 7.5-kbp species (Tn*4503*) (90). The 7.5-kbp transposon sequence includes a region that is homologous to the smaller transposons.

Further evidence that these inserted sequences are transposons is that the transposon probe also hybridized extensively with chromosomal DNA, showing that these sequences are also found in the chromosome. Also, these sequences have been found inserted into new positions on two different plasmids (21, 91). *C. taeniospiralis* 30-1 and 169-1 are derived from laboratory cultures of strains 30 and 169, respectively. Plasmids isolated from strains 30-1 and 169-1 differ in size from the parental plasmids. Analysis of these plasmids revealed that a 1.5-kbp sequence had been inserted into pKAP30, giving rise to pKAP30-1, and a 7.5-kbp sequence had been inserted into the R-body-encoding region of pKAP169, giving rise to pKAP169-1, which no longer directs R-body synthesis.

Each of the three transposons have been cloned in *E. coli*, although not as undisrupted sequences, and have been mapped by restriction endonuclease analysis (Fig. 17). The maps are highly homologous. Within Tn*4503* is a sequence that appears to be a truncated version of Tn*4501*. It is possible that Tn*4503* is a composite transposon in which Tn*4501* (or a closely homologous transposon) was inserted into a larger nonhomologous transposon (presumably about 6.0 kbp), resulting in a 7.5-kbp transposon. If the end of the smaller transposon was lost in the process, it would be fixed within this composite sequence. Tn*4502* differs from Tn*4501* in the distance between the *Stu*I and *Pst*I sites. An additional 100 bp is found in Tn*4502*. Otherwise, these smaller transposons are not distinguishable by their restriction maps. A question, as yet unanswered, is what protein products are coded for by these transposons?

Type 51 R Bodies Expressed in *E. coli*

The R-body-encoding genes from pKAP47 (the R-body-encoding plasmid from *C. taeniospiralis* 47) and pKAP116 (the R-body-encoding plasmid from *C. taeniospiralis* 116) have been cloned and expressed in *E. coli*. Quackenbush and Burbach (89) first cloned portions of pKAP47. Although none of their clones possessed killing activity, they were able to isolate clones that synthesized R bodies. Analysis of various subclones allowed them to determine the approximate location of the R-body-encoding sequences. They determined that the minimum DNA sequence needed to direct R-body synthesis was 1.3 kbp and that the maximum DNA sequence was 2.6 kbp. Also, because the orientation of inserted DNA did not affect R-body production, they concluded that the natural transcription signals were recognized in *E. coli* (i.e., transcription was not dependent upon transcriptional signals in the cloning vector). The DNA sequence required for type 51 R-body synthesis has now been placed at about 1.8 kbp and has been completely sequenced (Heruth, Ph.D. thesis) (see below).

The R bodies expressed in these clones are morphologically and behaviorally identical to those produced by *C. taeniospiralis*. Antibodies raised in rabbits against R bodies purified from transformed *E. coli* react positively with type 51 R bodies isolated from *C. taeniospiralis* 51, 116, and 169 (48). Additionally, R bodies from both sources possess the same appearance when viewed by electron microscopy (Fig. 18). They are ribbons with ends that form acute angles, typically coiled into a cylindrical form but capable of unwinding from the inside in response to lowered pH (89). Thus, R bodies expressed by the transformed cells are not appreciably different from native R bodies.

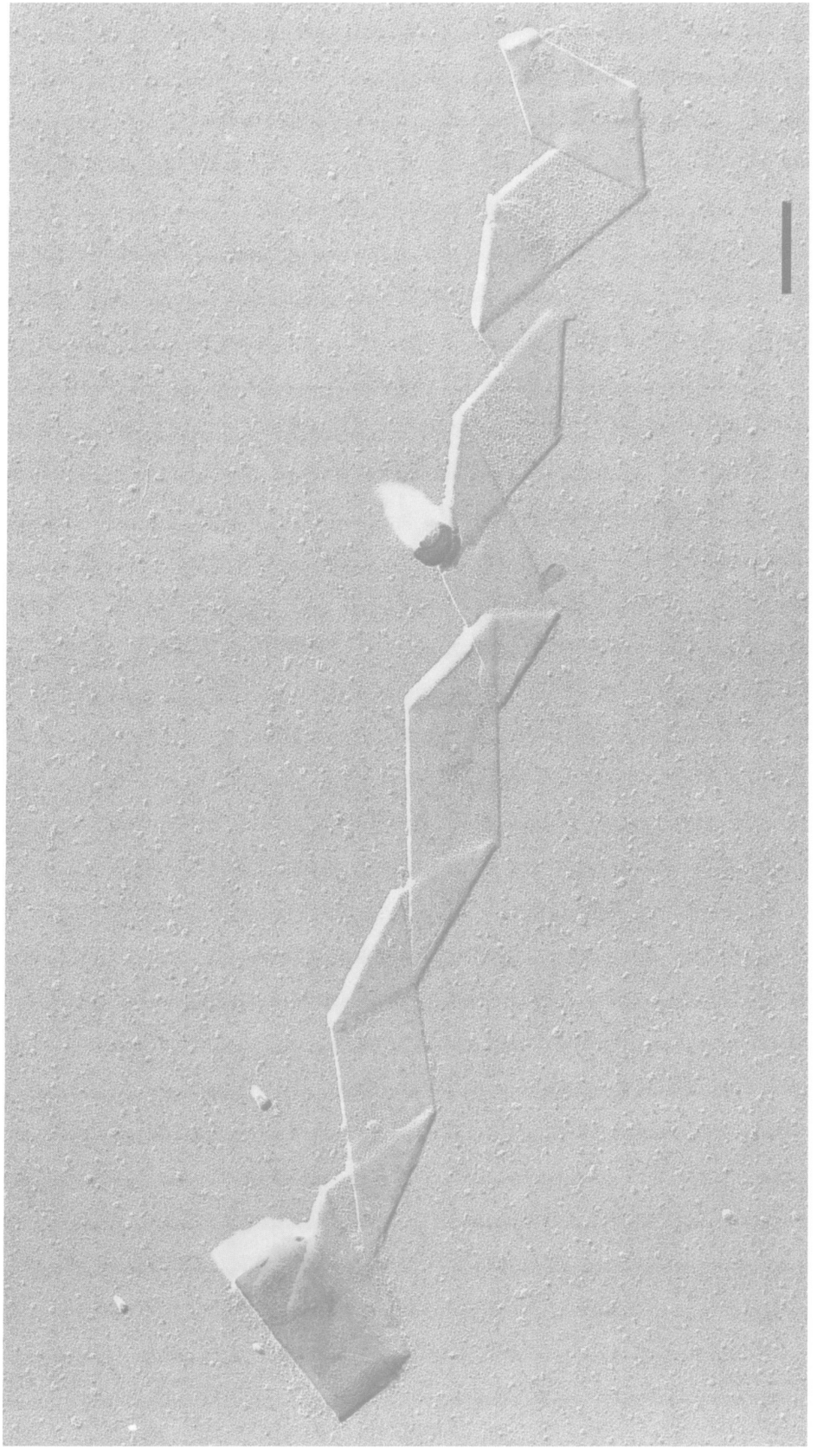


FIG. 15. Type 51 R body partially unrolled. Bar, 0.5 μm. Micrograph courtesy of B. Wells.

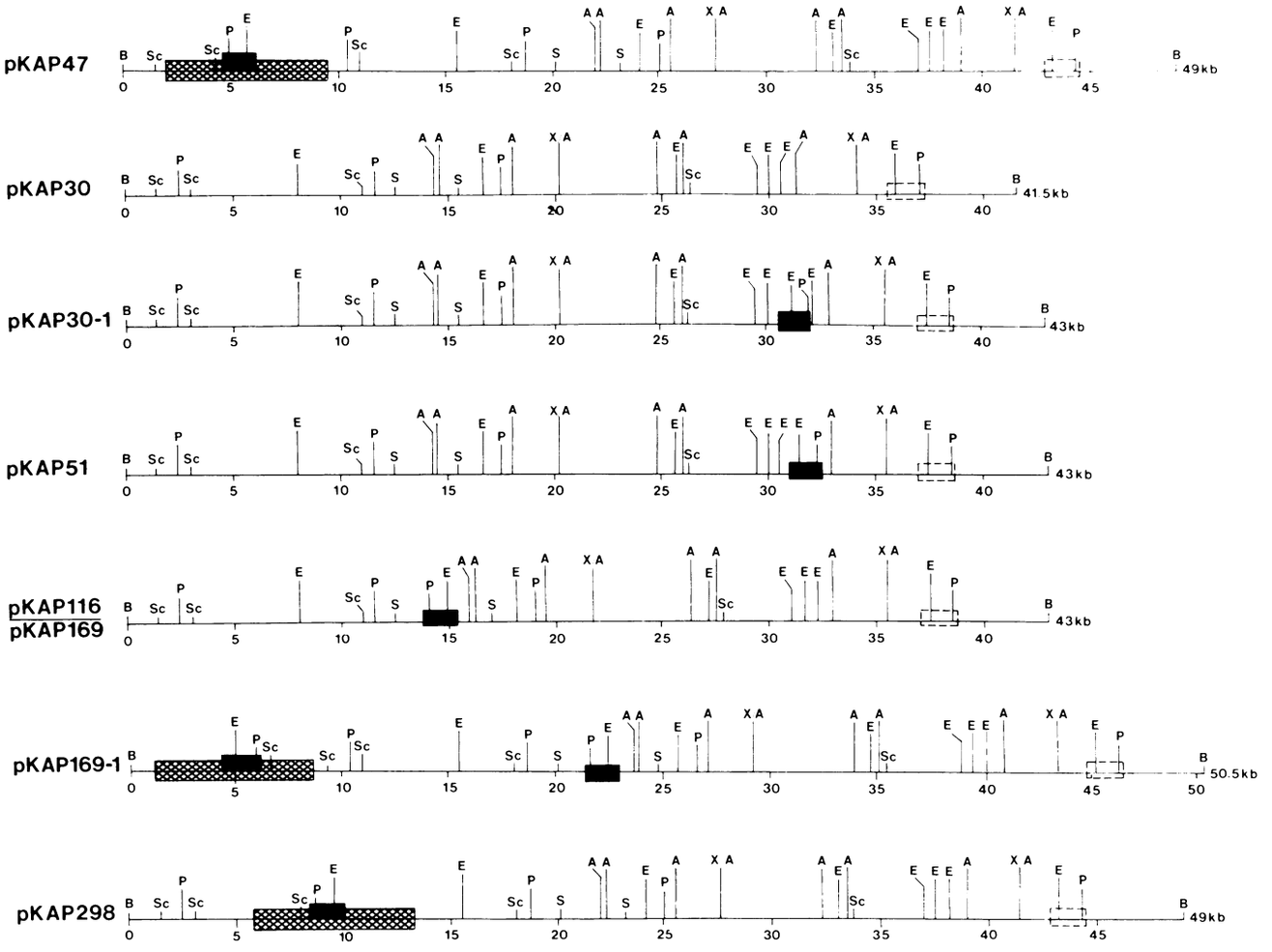


FIG. 16. Restriction maps of plasmids isolated from eight strains of *Caedibacter taeniospiralis*. Maps of pKAP116 and pKAP169 are identical and so are represented here by a single map. Symbols: ■, ▨, transposon sequences not present in pKAP30; ⁚⁚⁚, ca. 1.5-kbp transposon Tn*4501*, found in all plasmids; ■ in pKAP30-1 and pKAP51, Tn*4501*; ■ in pKAP116, pKAP169, and at position 22 in pKAP169-1, transposon Tn*4502*, which is also about 1.5 kbp and highly homologous with Tn*4501*; ▨ in pKAP47, pKAP298, and pKAP169-1, ca. 7.5-kbp transposon Tn*4503*; ▨, region homologous with Tn*4501* and Tn*4502*. Abbreviations for restriction endonuclease recognition sequences: A, *Ava*I; B, *Bam*HI; E, *Eco*RI; P, *Pst*I; S, *Sal*I; Sc, *Sst*I; X, *Xho*I. kb, Kilobase pairs. This figure is adapted from reference 91 with permission from the *Journal of Bacteriology*.

The homology seen in restriction maps of different *C. taeniospiralis* plasmids suggested that their R-body-encoding regions were also highly homologous, and, consequently, mapping the sequence homology between different *C. taeniospiralis* strains might provide insight into the functional regions of R-body structure. To this end, pKAP116 has also been cloned and expressed in *E. coli* (Heruth, Ph.D. thesis; F. R. Pond and D. P. Heruth, unpublished data). As expected, R-body synthesis was directed by the region corresponding to the R-body-encoding region in pKAP47. In contrast to pKAP47 clones, in which less than 3% of the bacterial population contained R bodies, the percentage of cells in pKAP116 clones containing R bodies was in some cases as high as 90%. R-body-encoding sequences from pKAP47 were inserted into pBR328 or pBR327 and introduced into *E. coli* 294. R-body-encoding fragments of pKAP116 were inserted into pUC18, and the recombinant plasmids were introduced into *E. coli* JM105 or JM107. When inserted into pUC18, the pKAP47 R-body-encoding sequence is also expressed at a high level in a variety of *E. coli* strains (including *E. coli* 294). The pUC vectors utilize a multiple cloning sequence near an efficient *lac* promoter, but transcription of the R-body genes in these recombinant plasmids proceeds opposite to the direction of transcription initiated at the *lac* promoter. Therefore, the high level of expression of these genes is not explained by their proximity to a strong promoter in the vector. Furthermore, the R-body-encoding genes in pBQ80 are removed from the *lac* promoter by more than 6 kbp. It is not known why high-level expression of the R-body-encoding genes occurs in the pUC vector.

Comparison of detailed restriction maps of pKAP47 and pKAP116 R-body-encoding regions predicts a high degree of homology between the R-body-encoding regions of pKAP47 and pKAP116 (Fig. 19). The sequence of the R-body-encoding region of pKAP116 has been determined (Heruth, Ph.D. thesis). No differences between the two sequences were observed.

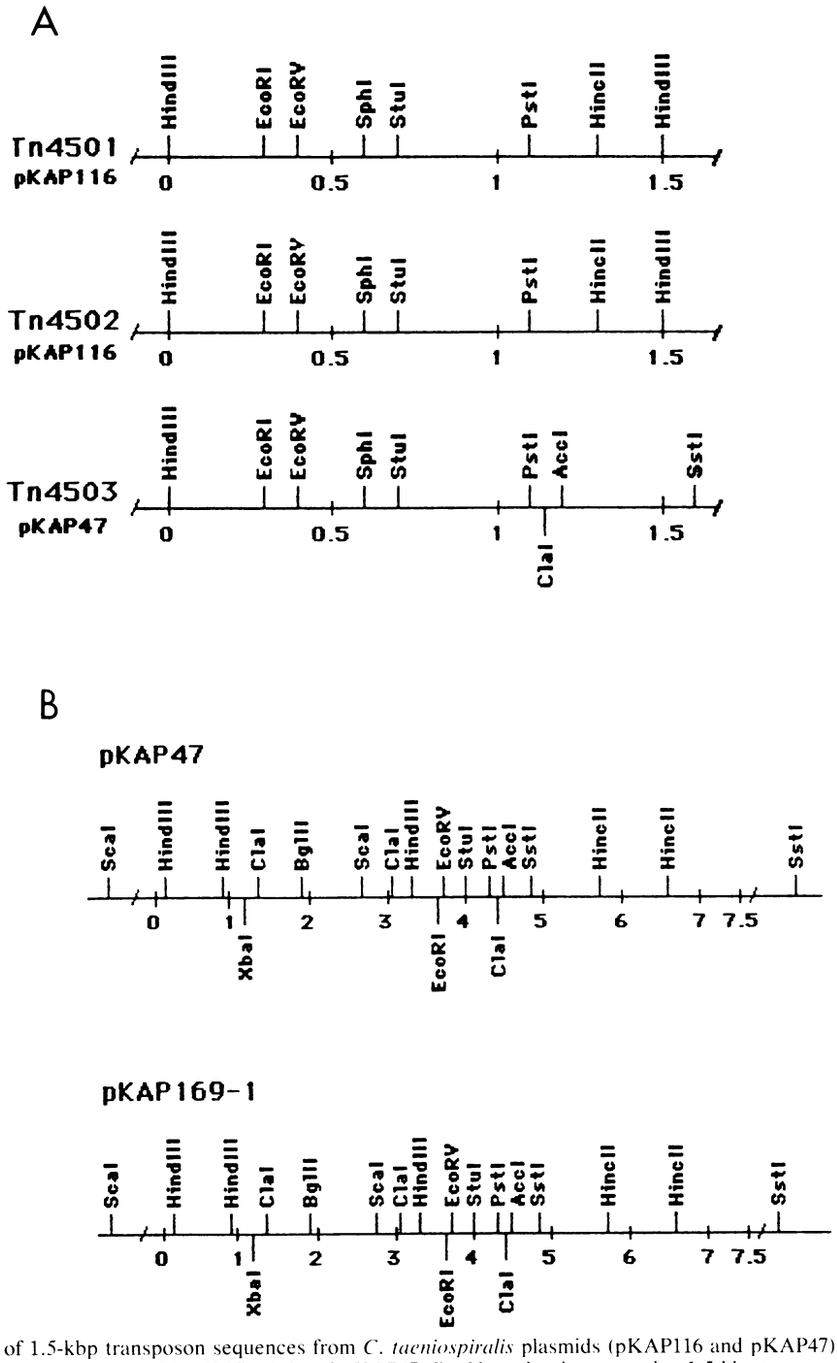
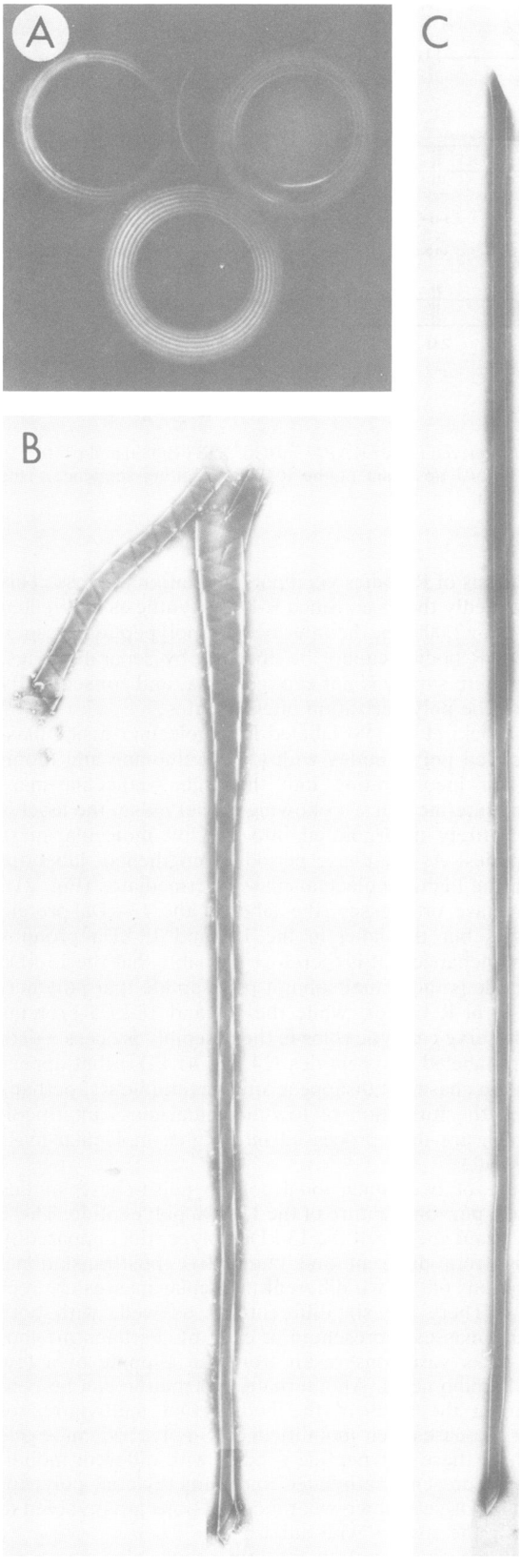


FIG. 17. Comparison of 1.5-kbp transposon sequences from *C. taeniospiralis* plasmids (pKAP116 and pKAP47) (A) and comparison of 7.5-kbp transposon sequences from plasmids pKAP169-1 and pKAP47 (B). Note that in comparing 1.5-kbp transposon sequences, only the central 1.5-kbp sequence from Tn*4503* (a 7.5-kbp transposon), homologous to Tn*4501* and Tn*4502*, is shown. This figure was provided courtesy of Susan Hodel.

Characteristics and Assembly of Type 51 R-Body Proteins

The assembly of R bodies is intriguing to consider. R bodies are large proteinaceous structures and, in the case of type 51 R bodies, appear to be synthesized from a single structural subunit. The close association with bacteriophages and bacteriophagelike structures has led to suggestions that R bodies may be aberrant assembly products of bacteriophages (76). Regardless of the truth of such speculations, understanding bacteriophage assembly processes may shed light on R-body assembly and vice versa. Certainly, such comparisons are informative.

Type 51 R bodies, whether isolated from *C. taeniospiralis* strains or from transformed *E. coli* (see above), are not completely dissociated by treatment under conditions known to denature other proteins (48). Treatments included incubation in combinations of 8 M urea, 1 to 10% SDS, and up to 5% 2-mercaptoethanol or dithiothreitol. Incubations were carried out at 100°C for up to 1 h. Additionally,



incubation at 37°C for 2 h in 8.6 M guanidine hydrochloride or 6 M guanidine thiocyanate, followed by dialysis and standard treatment with the final denaturing and reducing sample buffer, did not result in greater R-body dissociation. Also, boiling the R bodies for 5 min in dilute hydrochloric acid (pH 1.8) had no noticeable effect on dissociation. The unusual stability of these structures suggests that covalent bonds other than disulfide linkages are involved in the polymerization of subunit polypeptides, since it is unlikely that disulfide bridging, ionic or hydrogen bonding, and hydrophobic interactions are solely responsible for the resistance of R bodies to dissociation under the conditions described. Covalent linkage of proteins by other than disulfide bridges is probably not uncommon (36). Hendrix and Casjens (40) noted that covalent linkages, other than disulfide linkages, are involved in assembly of the bacteriophage lambda.

When purified R bodies are treated as described above and subjected to SDS-polyacrylamide gel electrophoresis, a characteristic pattern of polypeptide bands is generated (Fig. 20). Well over 30 polypeptide bands have been observed (48) ranging from about 10 to greater than 1,000 kDa (some protein is unable even to enter the stacking gel). The pattern of bands has been described as ladderlike for polypeptide bands above 40 kDa (48). Since this protein pattern is unaffected by denaturants, the polypeptide bands probably represent intermediates in the R-body assembly process rather than partially dissociated R bodies.

An odd feature of the R-body-associated polypeptides is that their migration in gels of different acrylamide percentages is not consistent relative to internal molecular weight standards (48). When the acrylamide percentage was increased, polypeptide migration was retarded relative to that of the molecular weight standards. Thus, estimates of molecular weights are quite uncertain. This phenomenon has not been explained. However, one possibility with respect to the oligomers is that they are linked in such a way that, under denaturing conditions, they require more space to pass through pores in a gel than do monomers of the same molecular mass; that is, under denaturing conditions, the oligomers have a width-to-length ratio closer to 1 than do monomers of the same molecular mass. Thus, as the pore size of a gel is decreased (i.e., the percentage of acrylamide is increased), the more difficult it would become for the oligomers to pass through relative to monomers of corresponding mass.

A series of experiments with the recombinant plasmid pBQ65, an R-body-encoding plasmid derived from pKAP47, showed that three (possibly four) polypeptides are involved in the assembly of R bodies (48). Three plasmid-encoded polypeptides, approximately 10, 13, and 18 kDa, were synthesized in vitro by using a coupled transcription-translation system (48). Assembly of the R-body subunits did not occur in this system. Self-assembly of certain viral structures does occur, although many viruses require assistance by host proteins for their assembly. It is not known whether host proteins are involved in the R-body assembly process, or whether in vitro self-assembly is possible under different conditions.

FIG. 18. R bodies isolated from *E. coli* that were transformed by a recombinant plasmid containing R-body-encoding sequences derived from pKAP47. (A) Untreated R bodies (pH 7.0); (B) partially unrolled R body with a smaller completely unrolled R body (pH 6.0); (C) completely unrolled R-body ribbon (pH 6.0), showing characteristically tapered ends. Bar, 0.5 μm. Reprinted from reference 89 with permission of the authors.

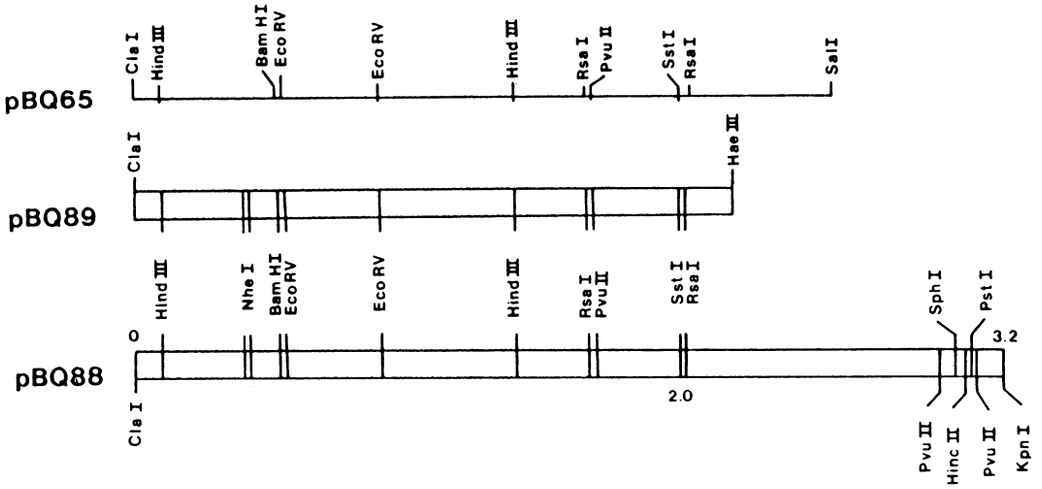


FIG. 19. Comparisons of restriction maps of cloned R-body-encoding sequences derived from pKAP47 (pBQ65 and pBQ89) and pKAP116 (pBQ88). The inserted sequences in pBQ65 and pBQ89 contain a portion of Tn*4503* upstream of the R-body-encoding sequence. This transposon is not present in pBQ88. This figure was provided courtesy of D. Heruth.

Plasmid-encoded proteins were also expressed in minicells (48), but in addition to the 10-, 13-, and 18-kDa proteins, higher-molecular-mass proteins were seen. When electrophoresed on SDS-polyacrylamide gels, these proteins give a pattern like that obtained from purified R bodies (with the addition of the β-lactamase protein coded for by the cloning vehicle portion of the R-body-encoding plasmid). These data were interpreted to mean that at least three polypeptides, encoded by the R-body-encoding sequence, are involved in the synthesis of R bodies via a polymerization process. This is undoubtedly the case. Since R-body synthesis is directed by less than 2,000 bp, the intermediate polypeptides associated with R bodies cannot be encoded by separate genes, because there simply is not enough DNA, and consequently they must be polymerization intermediates.

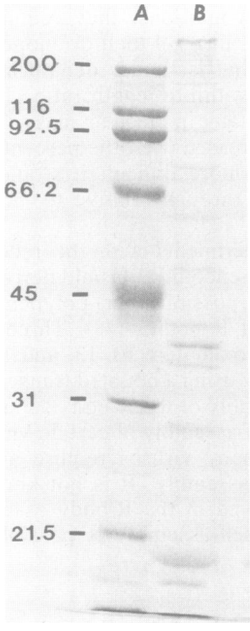


FIG. 20. SDS-polyacrylamide gel (7 to 20% gradient) showing proteins associated with purified type 51 R bodies. Lanes: A, molecular weight standards (in thousands); B, purified R bodies from *E. coli* P678-54(pBQ65). All samples were boiled for 5 min in final sample buffer before being applied to the gels. All gels were stained with Coomassie blue R-250.

Kanabrocki et al. (48) labeled low-molecular-mass, plasmid-encoded polypeptides with [^{35}S]methionine and monitored their incorporation into the higher-molecular-mass assembly intermediates. Following a brief pulse, the label is almost entirely incorporated into the low-molecular-mass polypeptides. As the chase period is lengthened, label appears in the higher-molecular-mass intermediates (Fig. 21). As the chase progresses, the label in the 13-kDa protein diminishes but the label in the 10- and 18-kDa proteins remains unchanged. It is therefore probable that the 13-kDa polypeptide is the actual subunit polypeptide that polymerizes to form R bodies, while the 10- and 18-kDa proteins probably serve catalytic roles in the assembly process. Also, there are labeled polypeptides (24 and 41 kDa) that appear early in the chase but disappear after several hours, perhaps reflecting the formation of low-molecular-mass intermediates which are then processed into higher-molecular-mass intermediates.

The use of two-dimensional gels in pulse-chase studies revealed a puzzling feature of the 13-kDa polypeptide. There are 12 variant species of the 13-kDa polypeptide, apparently resulting from different and cumulative posttranslational modifications (48). Two different molecular masses are represented. There are six different pIs as well, with both molecular masses represented at each pI. Perhaps the molecular mass variations result from the clipping of a few terminal amino acids. An alternate explanation can be constructed on the basis of the finding that methylation of proteins increases their mobility in SDS-polyacrylamide gels (17). Thus, these polypeptide species with different mobilities may represent methylated and unmethylated polypeptides. It is not yet known what modifications are involved in generating the species with different pIs.

During the chase period, the lower-molecular-mass species disappears first, but each of the six species with different

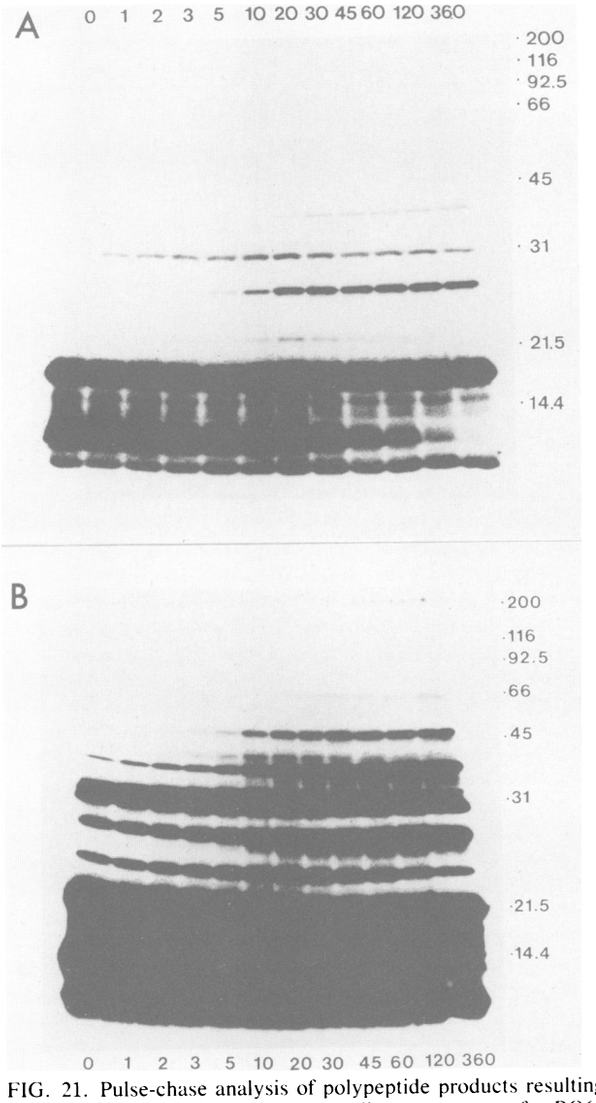


FIG. 21. Pulse-chase analysis of polypeptide products resulting from expression of the R-body-encoding sequence of pBQ65 (recombinant plasmid containing the R-body-encoding region of pKAP47). Minicells purified from *E. coli* P678-54(pBQ65) were labeled in the presence of [^{35}S]methionine for 3 min and chased with unlabeled methionine for the times specified (in minutes). The samples were boiled in final sample buffer for 5 min and resolved by electrophoresis on an SDS-polyacrylamide gel (7 to 20% gradient). Shown are fluorographs of the gel: (A) 12-h exposure; (B) 144 h exposure. Reprinted from reference 48 with permission of the *Journal of Bacteriology*.

pIs disappears at the same rate. Apparently, the trimming of amino acids from the 13-kDa polypeptide, or methylation of this protein, is required for polymerization, but the series of pI alterations either does not affect the assembly process at all or is required only at later stages of assembly, and so the alterations may take place before or during the process. Presumably, the 13-kDa gene product is subject to two independent modification processes.

While assembly of R bodies requires that the 10-, 13-, and 18-kDa polypeptides are present, partial assembly is seen when only the 10- and 13-kDa polypeptides are present (48) (Fig. 22). The role of the 10-kDa protein in this polymerization process is unknown. In the normal assembly process, these minor assembly intermediates, which are slightly basic, give way to slightly larger and acidic intermediates, which are apparently the major intermediates (Fig. 22). Note that as the polymerization progresses, the pIs shift toward the basic side. Also note that in both the major and minor intermediates, several pIs are represented. These pIs may reflect addition of different pI species of the 13-kDa subunit as assembly progresses.

Involvement of a fourth polypeptide in the assembly process has been postulated (48). There is ample space in the R-body-encoding sequence, about 500 bp between the sequences for the 10- and 13-kDa proteins, to code for a small polypeptide. An open reading frame has been identified in this region that would encode a polypeptide that can be easily detected in a minicell system labeled with [^{35}S]methionine (Heruth, Ph.D. thesis). No such polypeptide has been observed. This is consistent with the observation that no reasonable promoter sequences or ribosome-binding sites precede this open reading frame (Heruth, Ph.D. thesis).

The 18-kDa polypeptide is also represented by at least two molecular-mass variants. The 18-kDa protein does not seem to be involved as a subunit in the R-body assembly process but probably has a catalytic role. Although it is possible that either the 10- or 18-kDa polypeptide has an enzymatic role in the modification or covalent assembly of the 13-kDa protein, it is perhaps more likely that these polypeptides act as scaffolding proteins which direct the assembly process (see discussion of scaffold proteins in reference 39). It is also possible that covalent linkage of the 13-kDa subunits and their modifications may be host directed, although such processes are known to occur nonenzymatically (36).

Assembly of a protein ribbon such as an R body involves polymerization in at least two dimensions. In the structures considered thus far, the width of ribbons appears uniform for each R-body type. The length, however, varies, presumably with R-body maturity. In type 51 R bodies, polymerization of the 13-kDa subunit has not been seen when the 10-kDa polypeptide is absent. The 10-kDa polypeptide promotes polymerization of the so-called minor or shadow bands, but only in the presence of the 18-kDa polypeptide do the major polymerization bands appear. Apparently, the accessory polypeptides, the 10- and 18-kDa polypeptides, are involved in separate polymerization processes, and the two polymerization events may account for ribbon growth in different directions.

Because the width of R-body ribbons is uniform, assembly in this direction must be regulated. Kellenberger (50) summarized models by which bacteriophage tail length might be determined. One model, the cumulated strain model, postulates that subunits must be distorted during assembly; that is, the fit is somewhat forced. The strain that accumulates determines the limit of growth, since at some point the energy required for distortion exceeds the energy gained by bonding. In theory this is a plausible way to obtain a uniform tail length (124), but there is no experimental evidence that this mechanism regulates bacteriophage tail length. A second model, the vernier model, states that two proteins copolymerize into adjacent structures, but, because these polymers have different periods, they are out of phase with one another until reaching a certain length, at which time they are in phase and polymerization terminates. This model also is unsupported by direct experimental evidence. The third model, which has strong experimental support (39, 49), is the template or tape measure model. The length of bacteriophage lambda tails appears to be determined by a

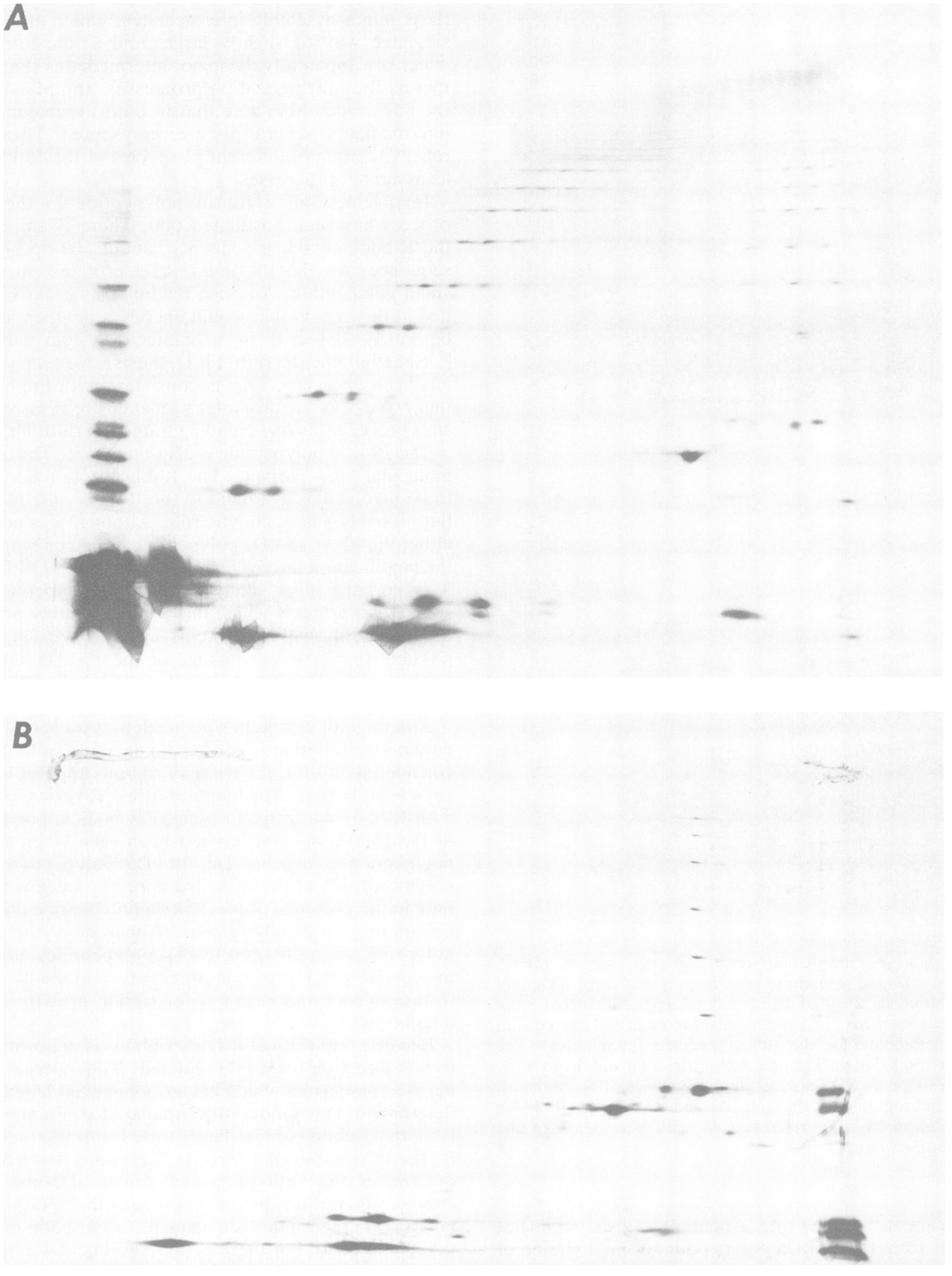


FIG. 22. Two-dimensional electrophoresis of proteins encoded by the recombinant plasmids pBQ89 (encoding the three polypeptides required for complete type 51 R-body assembly: 10, 13, and 18 kDa) and pBQ70 (encoding the 10- and 13-kDa polypeptides). Minicells containing the plasmids were labeled with [^{35}S]methionine for 45 min. The proteins were separated by isoelectric focusing and then separated in a second direction on an SDS-polyacrylamide gel (5 to 22.5% gradient). The basic pIs (+) are to the right, and the acidic pIs (−) are to the

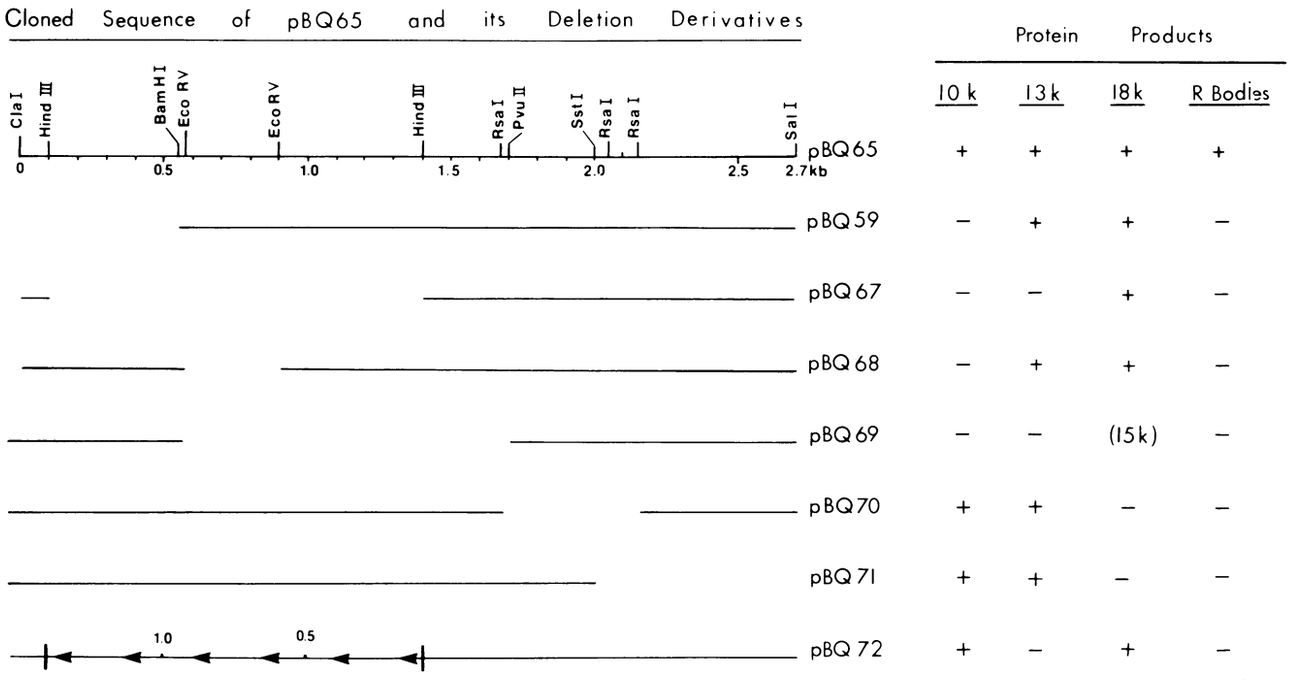


FIG. 23. Physical maps of the cloned sequences of pBQ65 and its deletion derivatives. Maps are shown with deleted regions appearing as gaps. The one exception is pBQ72, which is the result of inverting the 1.3-kbp *Hin*dIII fragment as indicated by the arrows above. Also included is a summary of the major polypeptide products encoded by each of these sequences. Reprinted from reference 48 with permission of the *Journal of Bacteriology*.

protein that actually spans the length of the mature tail and thus regulates the final length of the assembled structure.

Our understanding of R-body assembly is too preliminary to construct models for this process. Nevertheless, it is unlikely that a template model could specify ribbon width, since type 51 R bodies are 0.5 μm wide and the largest R-body-associated protein is less than 18 kDa. Thus, the ribbon is more than three times the length of lambda tails, while the likely candidates for a tape measure protein, the 10- or 18-kDa polypeptides, are four to eight times smaller than the lambda tape measure protein. It is possible that a variant of the cumulated strain or vernier models may explain ribbon width determination. Possibly the 10- or 18-kDa accessory proteins are involved in establishing the ribbon width by acting as a vernier. Alternatively, these accessory proteins may provide a scaffolding necessary for ordering or forcing the arrangement of the 13-kDa polypeptides. The consistently acute angles of the ribbon termini do suggest that ribbon growth follows an ordered assembly sequence which conceivably requires extensive scaffolding.

The studies of type 51 R-body assembly show that these R bodies, and presumably other R bodies as well, are constructed from unusual proteins and by an unusual process. It is not yet known to what extent the findings of studies with type 51 R bodies also apply to type 7 or type Cc R bodies. The observed differences in morphology and behavior suggest a difference also in the molecular components of type 7 and Cc R bodies, although the resistance of all kappa R bodies to dissociation suggests that they may share similar assembly processes.

Genetic Determinants for Type 51 R-Body Synthesis

Cloning the R-body-encoding sequence from the *C. taeniospiralis* R-body-encoding plasmid, pKAP47, has allowed the genetic determinants for this trait to be studied. Deletion derivatives and a sequence rearrangement of pBQ65 were constructed, and the polypeptides they encoded were analyzed in a minicell system (48; Heruth, Ph.D. thesis). In this way Kanabrocki et al. (48) were able to demonstrate the approximate location and order of the three genes that encode polypeptides necessary for R-body construction. Figure 23 presents restriction maps of the deletion derivatives and the one sequence rearrangement studied along with the polypeptides they encode. The order of genes are, from right to left as drawn in Fig. 23, the sequences coding for the 18-kDa polypeptide, the 13-kDa polypeptide, and the 10-kDa polypeptide. Note that R-body synthesis does not occur if any one of the three polypeptides is absent.

left. The cloning vector encodes β-lactamase, which can be detected as two species (molecular masses 28 and 30 kDa) in the basic edge of each gel. (A) Two-dimensional electrophoresis of proteins encoded by pBQ89. The standard lane (left) contains a one-dimensional profile of minicell-encoded proteins from pBQ89. Note the characteristic ladderlike profile. Two or three species of the 18-kDa protein exist. Two species of the 13-kDa polypeptide exist, each with at least six pIs. Two polymerization events are occurring. One polymerization event is basic, and the other polymerization event starts out acidic and becomes progressively more basic as the molecular masses of the polymerization products increase. Several species of proteins differing in pI can be identified at each level of polymerization. (B) Two-dimensional electrophoresis of proteins encoded by pBQ70. The standard lane (right) contains a one-dimensional profile of minicell-encoded proteins from pBQ70. Only the basic polymerization event can be detected. The 13-kDa protein exists as two molecular mass species with several pIs. This figure represents previously unpublished data and was provided courtesy of D. Heruth and F. Pond.

In pBQ71, the end of pBQ65, right of the *Sst*I site, has been deleted (Fig. 23). This eliminates production of the 18-kDa polypeptide. In another deletion derivative, pBQ69, a large (about 1.1-kbp) region was removed (Fig. 23). This plasmid coded for a 15-kDa polypeptide which is a truncated form of the 18-kDa polypeptide. Thus, the regulatory sequences for the 18-kDa polypeptide are located to the right of the *Sst*I site (as drawn in Fig. 23), and transcription is in the leftward direction. Since deletion of this regulatory region affects only the expression of the 18-kDa protein, it was concluded that each gene is expressed as an independent transcriptional unit.

Two R-body-associated polypeptides, the 13- and 18-kDa polypeptides, have been determined by two-dimensional gel electrophoresis to actually be groups of polypeptides. The 13-kDa group consists of at least six species on the basis of pI, with each species consisting of a pair of polypeptides with slightly different molecular masses. The 18-kDa group consists of three species with different molecular masses but all exhibiting the same pI. Since the size of the DNA sequence that directs R-body synthesis is less than 2,000 bp and deletion derivatives affect either all the species in one of these groups or none of the species, Kanabrocki et al. (48) concluded that there are single translation products, approximately 13 and 18 kDa, which are subjects of an as yet unidentified series of posttranslational modifications.

DNA sequencing data (Heruth, Ph.D. thesis) confirm the existence of three transcriptional units. Calculations based on the sequencing data indicate that the actual sizes of the three polypeptides involved in R-body synthesis are only about 60% of the estimated sizes based on SDS-polyacrylamide gel electrophoresis. The sequencing data also demonstrate that transcription and translation signals are similar to those of *E. coli*. The same is also true of codon usage in the three open reading frames that code for the three polypeptide species required for R-body assembly.

R Bodies and Toxicity

The observation that toxicity was associated with bright particles served to narrow the focus of attention to the relation between R bodies and toxicity. However, attempts to elicit killing activity with purified R bodies have resulted in only limited success. Killing activity has been achieved by using purified type 7 R bodies from certain *C. varicaedens* strains (80, 81). Killing has not been demonstrated with type 51 R bodies.

Type 7 R bodies. It is not understood why certain R bodies possess killing activity after being purified, whereas others do not. Type 7 R bodies, which, when purified, are capable of killing sensitive paramecia, are nearly always surrounded by a membranous sheath (80). These R bodies also tend to be associated with capsomerelike structures, which, along with bacteriophages, adhere to the R-body ribbons (80). Type 7 R bodies purified from strain 562 are an exception, since they exhibit weak killing but are not enclosed by a sheath, nor are capsomeres present. Furthermore, capsomeres are associated with R bodies in *C. pseudomutans* 51m1, but these R bodies lack killing activity when purified. Thus, the presence of a membranous sheath around the R bodies and the presence of capsomeres do not consistently correlate with toxicity. It is always the case, however, that killing occurs only if the R bodies have not unrolled. The importance of this is not known.

From these observations, one cannot determine whether the toxin is an integral part of these R bodies, is simply being delivered to its target site by the R-body structure, or is associated with the bacteriophages or capsomerelike structures. It is possible that the toxin, as the cargo of the R body, is simply jettisoned when the R body unrolls. However, it is equally possible that treatments used to cause the R bodies to unroll also inactivate the toxin.

It has been claimed that R bodies and their associated bacteriophages are serologically cross-reactive with each other and that these structures are related to the toxin. This was inferred from the observation that toxicity of purified type 7 R bodies is blocked by pretreating these R bodies with antisera raised against either the R bodies themselves or their associated bacteriophages (76; M. J. Singler-Bastiaans, Ph.D. thesis, Indiana University, Bloomington, 1975). These observations have not, however, been confirmed. Nevertheless, the ability of these antisera to block toxicity suggests that the toxin is a component of R bodies (or is, at least, adsorbed to them). It is not known why activity should be limited only to wound R bodies, but, apparently, in some way, unrolling of the R bodies may be involved in toxicity. The contrasting hypothesis that R bodies are not themselves toxic or even directly involved in toxicity, but instead act only as vehicles for transporting the toxin, is supported by the inability of either rolled or unrolled R bodies from other strains to kill sensitive paramecia.

We must mention at this point (in light of the possible relationship of bacteriophages to R bodies) that the bacteriophages and capsomeres associated with R bodies have also received attention as possible toxic agents (76). However, Preer et al. (76) argue that phage multiplication is unlikely to contribute to toxicity because the onset of symptoms is too rapid. Furthermore, these bacteriophages are believed to be defective, i.e., noninfective. Additionally, bacteriophage and capsomere proteins are not likely candidates for the toxic agent, since their presence or absence is not correlated with the presence or absence of killing activity.

Type 51 R bodies. In *C. taeniospiralis*, R-body synthesis is known to be directed by genes carried on a plasmid. Because the R bodies are always associated with toxigenicity, both traits appearing simultaneously, it has been assumed that toxin is also encoded by plasmid genes and that both traits (R-body and toxin production) are induced simultaneously. Purified type 51 R bodies possess no toxic activity, but toxicity has not been detected for any kappa strain lacking R bodies. The question, then, is whether R bodies, although not toxic themselves, are required for expression of the killer trait or are merely associated with toxicity by coincidence.

Observations of mutant kappa strain 51m43, generated by X-ray treatment, indicated that killing activity was linked to R-body production (127). The mutant phenotype, which exhibited very weak killing and a very low frequency of R-body production (although resistance to killing was retained), was apparently associated with changes in plasmid replication (20). Dilts (20) showed that this mutant had several plasmids (28, 57, 94, and 114 MDa). The 94-MDa plasmid was apparently a minor plasmid unrelated to the R-body-encoding plasmid (92). However, the 57- and 114-MDa plasmids were presumably polymers of the 28-MDa R-body-encoding parent plasmid. These large plasmids were found in low copy numbers. It was suggested (21, 84) that the 51m43 mutation involved plasmid replication, either directly or indirectly, such that there was reduced expression of plasmid-encoded traits (e.g., R-body and toxin production). Unfortunately, all cultures of this mutant strain have been lost, and so its mutant plasmid forms cannot be analyzed in

light of what is currently known about the R-body-encoding sequence in *C. taeniospiralis* plasmids. Nevertheless, these observations are compatible with the view that both the toxin and R bodies are plasmid encoded and that both are required for killing activity.

Dilts and Quackenbush (21) have provided convincing evidence that R bodies are required for the killing trait to be expressed. They describe a mutant strain (169-1) of *C. taeniospiralis* 169 which simultaneously lost the abilities to produce R bodies and to kill sensitive paramecia. This was a spontaneous mutation of a laboratory culture. Investigations of the R-body-encoding plasmid isolated from the mutant *C. taeniospiralis* 169-1 revealed that a 7.5-kbp sequence had been inserted within the R-body-encoding region (Fig. 16), thereby eliminating R-body production. A restriction map was constructed for pKAP169-1 (the R-body-encoding plasmid from the mutant strain *C. taeniospiralis* 169-1) and compared with the map for pKAP169. The inserted sequence in pKAP169-1 is identical in size and restriction sites to Tn*4503*, which is found in pKAP47 and pKAP298 (21, 91). A radioactive DNA probe, including about two-thirds of Tn*4503* from pKAP47, was made by nick translation. Hybridization of the probe DNA to Southern blots of restriction endonuclease-digested pKAP169-1 DNA demonstrated that the inserted DNA is homologous to Tn*4503* from pKAP47. Previous hybridization studies had shown that some *C. taeniospiralis* strains carried chromosomal (endosymbiont) DNA sequences homologous with Tn*4503* (91). Therefore, it was concluded that the DNA insertion into pKAP169 (forming pKAP169-1) represents the transposition of Tn*4503* from chromosomal DNA to plasmid DNA. This clearly explains the loss of R-body synthesis in *C. taeniospiralis* 169-1.

Type 51 R bodies are not themselves toxic to sensitive paramecia (80, 89). However, since the killer trait is absent from the mutant strain 169-1, it is reasonable to conclude that R bodies are necessary, although not sufficient, for the killer trait to be expressed.

Gibson (I. Gibson, unpublished data) reported that a 20- to 25-kDa protein isolated by fast protein liquid chromatography from cultures of killer paramecia (*P. tetraurelia* 51) possesses toxic activity against sensitive paramecia. He noted that blistering and death of sensitive paramecia occurred within 3 days of exposure to this protein and that the minimum unit of activity was approximately 20 pg/ml. However, toxic activity could not be isolated from cultures of other killer paramecia (stocks 540, 138, and 7). Gibson and Wells (I. Gibson and B. Wells, unpublished data) reported that there is no immunological cross-reactivity between the isolated toxin and R-body proteins. Consequently, the role of R bodies in the killing process remains unclear. Gibson (unpublished) suggested that toxin may be a by-product of the biochemical pathway producing R bodies or vice versa. The significance of these findings remains to be determined, as does the relationship of this toxin with type 51 R bodies. Localization of toxin on R-body-producing bacteria but not R-body-free bacteria would be a convincing proof of a relationship between R body and toxin.

Mechanism of killing. Different strains of killer paramecia cause sensitive paramecia to die in different ways. However, although the toxins may vary in some ways, there are common features in most types of killing.

Killer paramecia release kappa particles into their environments via the cytopyge (67, 76), and sensitive cells are killed after ingesting a single bright particle (5). Once ingested and incorporated into a food vacuole, the bright particle is digested, triggering a conformational change in the R body that is apparently important for toxin release (76). In kappa particles, the R body is a tightly coiled protein ribbon (3). However, Jurand et al. (45, 46) observed, in sensitive paramecia exposed to isolated kappa particles, unrolled R bodies penetrating food vacuole membranes, extensive membrane breakage and disintegration, and vacuole contents mixed with cytoplasm. They also noted that food vacuole formation ceased shortly after exposure to bright particles. Except for *C. paraconjugatus*, a mate killer, in which physical contact between killer and sensitive paramecia is generally thought to be required for killing to occur, toxin probably enters paramecia in the same manner regardless of the kappa strain from which it is derived.

Clearly, R bodies are necessary for killing activity, apparently transmitting toxin to its target site. However, the nature and mechanism of action of the toxin have yet to be elucidated. Preer and Preer (74) have, however, reported evidence that the toxin is proteinaceous. They treated isolated type 7 R bodies with chymotrypsin and then tested them for killing activity; they found that paralysis killing had replaced spin killing. Jurand et al. (46) compared prelethal effects of hump killing, spin killing, and vacuolization and suggested that the toxins are related in that they affect osmoregulatory properties of biological membranes. Kappa toxins remain to be isolated and more fully characterized.

Early efforts to quantitate toxicity led to the conclusion that a single toxin particle is sufficient to kill a sensitive paramecium (5), and that toxin particles are clearly equivalent to bright kappa particles (66, 78). However, although killing activity is associated with bright kappa particles, toxin particles are not identical to R bodies (see above discussion). Therefore, quantitation of the toxin may have little to do with numbers of R bodies.

Sonneborn (110) noted that the number of toxic particles in killer paramecium homogenates is only about 1% the number of bright particles. How is this observation to be explained? Are all bright particles toxic but the probability of a bright particle being ingested by a sensitive paramecium very low, resulting in an underestimation of the number of toxic particles? Do only some bright particles acquire toxicity? Is toxicity lost as bright kappa particles age? Perhaps toxicity is ameliorated by such factors as the cell stage during which sensitive paramecia are exposed to the toxin or the presence of toxin-resistant paramecium homogenate. Sonneborn (110) proposed that maturation of bright particles is required for expression of toxicity and cited as evidence the observation that the toxic potential of killer homogenates increases if they are allowed to "age" for 1 to 2 hours. The meaning and significance of these observations are not known.

When homogenates of killer paramecia are treated with certain biochemical inhibitors, the toxicity is altered. Butzel and Pagliara (13) found that sodium azide and dinitrophenol decreased toxicity, implying that the maturation process depends upon oxidative metabolism. The effect of dinitrophenol was blocked by adding adenosine triphosphate simultaneously. Apparently, the formation of lethal particles is not dependent upon protein synthesis, since chloramphenicol-treated homogenate did not exhibit reduced killing activity. Chloramphenicol treatment of sensitive paramecia, however, did provide some protection. This may have been an indirect effect of shutting down mitochondrial activity. Malonate and sodium fluoride, which poison the Krebs cycle, as well as xanthine, were shown to increase the toxicity of homogenates. These findings have not been satisfactorily

explained. It is possible that bright particles contain a protoxin that is activated by adenosine triphosphate-dependent reactions whose regulation is tied to, or modified by, Krebs cycle activity.

Other studies have shown that high temperatures (above 55°C), extreme pH, and certain proteolytic enzymes also inactivate toxicity in killer homogenates (110). Deoxyribonuclease has been reported to decrease toxic activity of killer homogenates (122), although this was disputed (100, 110). The significance of these findings is obscured, since it is unclear whether these are direct or indirect effects.

Butzel et al. (12) demonstrated that lecithin protects sensitive paramecia from the killing activity of stock 51 killer paramecium homogenate. The protection was dose related and was nearly complete at the optimal dosage. Sensitive paramecia, rather than homogenate, were apparently affected by lecithin treatment, since the protective effect is seen even when the sensitive cells are first allowed to ingest untreated particles from the killer homogenate and then are transferred to lecithin-containing medium. Jurand et al. (46) reported that food vacuole formation ceases shortly after the sensitive paramecium ingests a bright kappa particle. It is questionable, then, whether the phospholipid is being incorporated into the cell, since it is not known whether pinocytosis is also affected. Butzel et al. (12) believed that lecithin exerted its prophylactic effect at the cell surface. It is interesting that Jurand et al. (46) noted numerous lipid droplets in the cytoplasm of sensitive cells after exposure to stock 51 kappa symbionts. They postulated that these droplets resulted from membrane decomposition. It is possible that lecithin protects sensitive cells not by binding or inactivating toxin but by promoting membrane integrity in the face of toxin attack. However, the availability of lecithin for this purpose, under the experimental conditions described by Butzel et al. (12), is uncertain. It is also interesting that choline provided no protection and cephalin (24) provided only partial protection.

The work of Jurand et al. (46) suggested that the target sites of kappa toxin are biological membranes, and Heckmann (personal communication) has acquired evidence that kappa 562 toxin has an affinity for membrane components. He exposed sensitive cells to various concentrations of kappa 562 bright particles. A direct correlation was seen between the kappa particle concentration and the number of sensitive cells that died after a 4-h period. When *N*-acetyl-D-galactosamine was added to the culture fluid, the sensitive cells were protected. By varying the sugar concentration, it was demonstrated that the degree of protection offered was dose related. Other sugars were tested but were less effective than *N*-acetyl-D-galactosamine in preventing toxicity. These findings suggest that the sugar binds to the toxin, thereby inactivating the toxic effect. Although the character of the toxin and its mechanism of action remain a mystery, such observations as described above are intriguing and suggest that the structure and function of membrane components be studied more closely in relation to toxic activities.

R-BODY-CONTAINING PSEUDOMONADS

P. taeniospiralis

Background. During morphological studies on hydrogen-oxidizing bacteria isolated from soil samples from a soccer field in Barcelona, Spain, one strain (2K1) of bacteria aroused attention because some of these cells contained unusual intracytoplasmic membrane structures referred to as spiral bodies (54). This bacterium represented a new pseudomonad species and was named *P. taeniospiralis* (56). Until the discovery of these spiral bodies, R bodies were believed to be unique to members of the genus *Caedibacter*. However, Lalucat et al. (55) demonstrated that these spiral bodies exhibit the essential characteristics of R bodies; they are protein ribbons coiled into a cylinder but capable of unrolling. The studies of Lalucat et al. also showed differences between the R bodies in *P. taeniospiralis* and kappa R bodies. Thus, a new class of R body was discovered. This discovery was particularly exciting because it was the first indication that R bodies are not unique to endosymbionts of paramecia, but are also found in free-living bacteria. This discovery also generated important questions pertaining to R-body evolution and function.

P. taeniospiralis is a hydrogen-oxidizing, R-body-containing bacterium further characterized as a gram-negative, yellow-pigmented cell with a polar flagellum and a tendency to pleomorphism (53, 54, 56). It is a facultative chemolithotroph. This organism is notably distinct from other hydrogen-oxidizing bacteria in its flexibility as a heterotroph, since it can utilize a wide variety of sugars and many organic acids as its sole carbon source (56).

***P. taeniospiralis* R bodies.** The R bodies produced by *P. taeniospiralis* (type Pt R bodies) are distinct from other R-body types. Type Pt R bodies are comparatively small (55). Unrolled R-body ribbons average 0.21 μm in width and 6.0 μm in length, and the diameter of the coiled ribbon is about 0.25 μm (Fig. 24). Type Pt R bodies unroll from the inside in a telescopic fashion, as do type 51 and type Cm R bodies. Unrolling is irreversible for type Pt R bodies and does not occur in response to pH changes. These R bodies do, however, unroll quite readily when incubated at 65°C for 5 to 10 min (31). The inner terminus of the R-body ribbon (at the center of the coiled R body) is acutely tapered, whereas the outer end is blunt. There is no sheath surrounding these R bodies, nor do bacteriophagelike structures adhere to the ribbons, as is the case for certain type 7 R bodies. Like type 51 R bodies, dissociation of type Pt R bodies does not occur under conditions known to denature or solubilize proteins (J. Lalucat, personal communication), implying that covalent linkages are involved in the assembly of the type Pt R-body structure. R-body production in *P. taeniospiralis* is highest in the stationary growth phase, with up to 43% of the bacterial population containing R bodies, although cells containing R bodies are found during all growth phases and under both autotrophic and heterotrophic culture conditions (55). Frequently, more than one R body is seen per cell in the stationary growth phase. In recent studies (J. Lalucat and J. Imperial, Micron Microsc. Acta **18**:37–40, 1987), R-body production has been reproducibly induced at a high level (greater than 40% of the bacterial population containing R bodies) when gluconate serves as the sole carbon source.

Genetic determinants for R-body production in *P. taeniospiralis*. Lalucat et al. (55, 56) suggested that R-body production in *P. taeniospiralis* is determined by a defective prophage. Structures resembling bacteriophage heads, although quite rare, were observed in a suspension of R bodies (55). Additionally, exposure to mitomycin C, a treatment that induces prophages, results in the appearance of bacteriophage taillike structures. Thus, these cells appear to be infected by a defective prophage. The analogy to *Caedibacter* species is striking. In *Caedibacter* species, R bodies are encoded by extrachromosomal elements; type 51 R bodies are clearly encoded by plasmids, and the evidence strongly

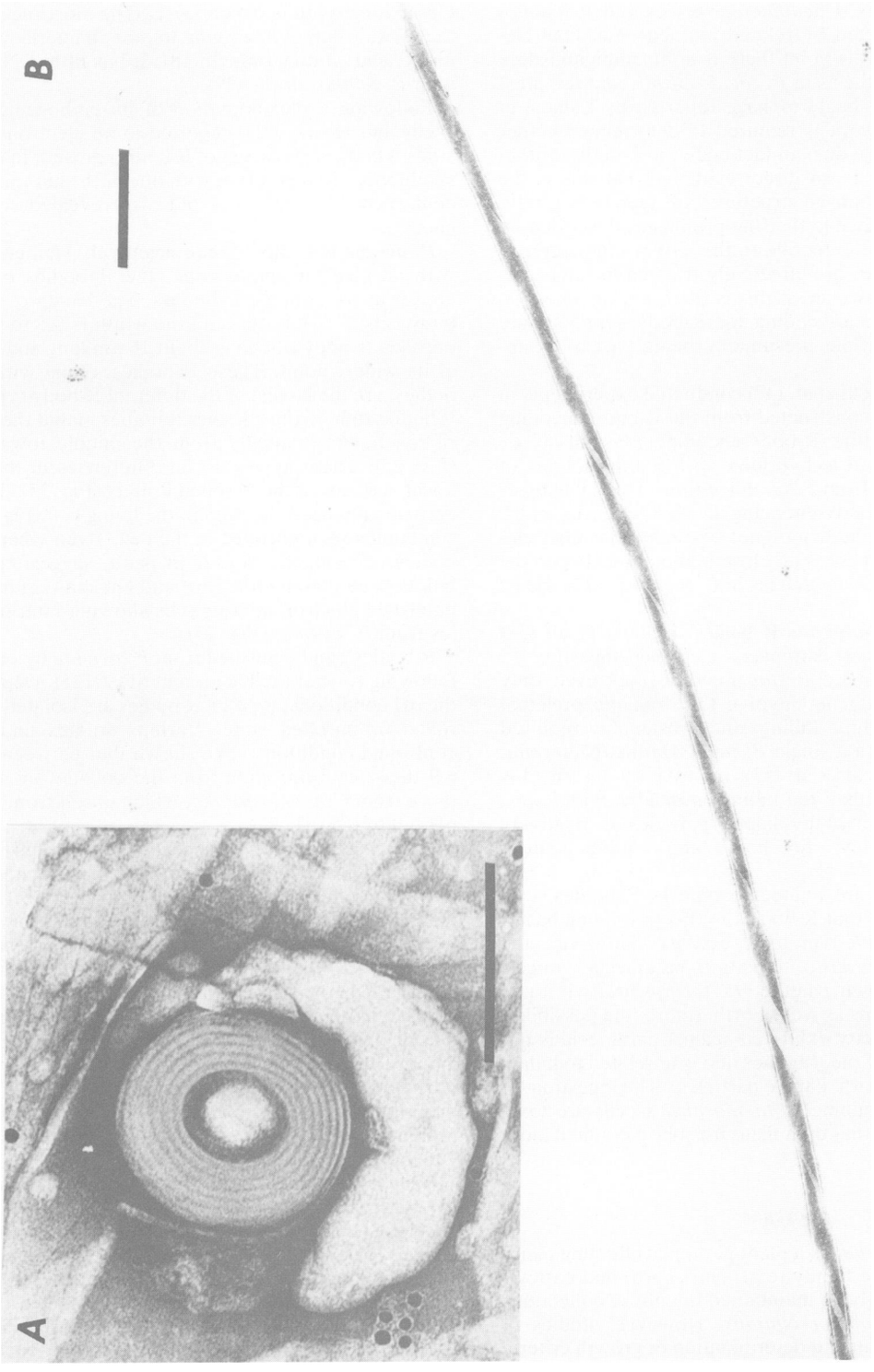


FIG. 24. Type Pt R bodies. (A) Coiled R body in cross section (bar, 0.2 μm); (B) unrolled and fully extended R body (bar, 0.5 μm). Electron micrographs courtesy of B. Wells and B. Bird.

favors the hypothesis that defective bacteriophages encode type 7 R-body synthesis.

If, as suspected, type Pt R bodies are encoded by a defective bacteriophage, then one can more easily imagine a common origin for kappa R bodies and the R bodies of *P. taeniospiralis*. That is, *Caedibacter* species and *P. taeniospiralis* may be infected by related extrachromosomal elements. However, at this point there is no unambiguous link between R-body synthesis in *P. taeniospiralis* and the presence of the defective bacteriophage reported by Lalucat et al. (55). Additional work is required to determine whether the defective prophage does in fact code for R-body synthesis. At present there is no direct evidence that this is the case, and the bacteriophage structures are seen only rarely. However, it is conceivable that the prophages described by Lalucat et al. (55) are defective to the extent that bacteriophage DNA sequences are effectively trapped in the bacterial chromosome. However, if this is the case, the question arises of how sequences coding for R-body synthesis are selectively induced while presumably being part of a suppressed prophage.

Recently, Kanabrocki et al. (47) conducted experiments in which a DNA probe constructed from the R-body-encoding region of pKAP47 (the R-body-encoding plasmid of *C. taeniospiralis* 47) failed to hybridize with Southern blots of total cell DNA taken from *P. taeniospiralis*. Thus, whatever the origin of the R-body-encoding DNA sequences of *P. taeniospiralis*, i.e., whether or not extrachromosomal elements are involved, there is no close homology between the R-body-encoding DNA sequences in *C. taeniospiralis* and *P. taeniospiralis*.

Toxicity of *P. taeniospiralis* R bodies. Lalucat et al. (53) reported that sensitive paramecia die after ingesting *P. taeniospiralis*. Killing by *P. taeniospiralis* occurred only after several days, did not involve the dramatic prelethal symptoms seen in kappa killing, and presumably required ingestion of more than a single *P. taeniospiralis* bacterium. In the study by Lalucat et al. (53), the toxicity described is considerably weaker than the killing associated with kappa particles. Killing elicited by kappa particles is relatively quick, occurring in only a few hours, and requires a single bright kappa particle (4, 5).

If kappa R bodies are related to type Pt R bodies, it is certainly conceivable that R bodies in this free-living bacterium might be involved in toxic activity. However, the benefit to *P. taeniospiralis* of toxicity to paramecia is uncertain, and it is unknown whether *P. taeniospiralis* is toxic toward other organisms as well. Furthermore, the possibility remains that the toxicity exhibited against paramecia is not due to the presence of the R bodies and is unrelated to killing activity associated with kappa particles. The question of whether R-body-containing *P. taeniospiralis* cells are toxic to paramecia or any other organisms must be examined more closely.

P. avenae

Background. *P. avenae* is a plant pathogen affecting members of the Gramineae family (e.g., oats, corn, and barley). This species has also been maintained in culture collections under the name *P. alboprecipitans*. However, studies of DNA base composition and determination of growth criteria have shown that the two organisms are identical (93). *P. avenae* cells are 0.6 μm wide by 1.6 μm long, gram-negative, polar-flagellated rods. They require oxygen for growth, are nonfluorescent, and reduce nitrate aerobically. These bacteria have little DNA homology with other pseudomonads that are plant pathogens. Different isolates of *P. avenae* have a base composition of 67 to 71 mol% G+C (18, 93). Older colonies are sticky, adhere to agar, and produce a slime which makes purification of subcellular components difficult. Optimum growth occurs at 37°C. The most recent studies of the relationship of *P. avenae* to other bacteria have placed it in the acidovorans branch (18). It has little DNA homology with *P. taeniospiralis* (29).

Following a reexamination of its pathogenic properties, Wells and Horne (125) reported in an electron-microscopic study a body reminiscent of R bodies present in *Paramecium* symbionts. Collaboration with one of the authors (I. Gibson) confirmed the similarities but also revealed several differences.

***P. avenae* R bodies.** When negatively stained and viewed with an electron microscope, the R bodies of *P. avenae* appear as asymmetric cylinders. The length of the coiled R body is 0.75 to 0.8 μm, and the width is 0.5 to 0.8 μm. The unrolled R-body ribbon is about 16 μm long and 0.8 μm wide at its widest point. These R bodies, along with type Cc R bodies, are the largest of the different R-body types. Type Pa R bodies differ from all other R bodies in that the width of the ribbon tapers gradually from the middle toward the ends (Fig. 25). There appear to be differences in the upper and lower surfaces of the unrolled R body (Fig. 25). The R bodies occasionally occur in pairs in the living cell (Fig. 26) and are sometimes seen unrolled in the cell. Even when isolated, R bodies occasionally appear in pairs, suggesting a physical link between them (10). Bird and Gibson (11) have recently published electron micrographs showing interlinked R bodies from *P. avenae* (Fig. 27).

R bodies can be purified from *P. avenae* by centrifugation following lysis of the bacteria in SDS (125). Depending upon the pH conditions, type Pa R bodies are isolated in either the rolled or unrolled state. Various studies under carefully controlled conditions have shown that between pH 6.8 and 6.9 they begin unrolling from the outside, in contrast with those from *C. taeniospiralis*, which unroll from the inside at pH 6.0 to 6.5 (63, 72).

Other variables that reportedly affect the unrolling of type Pa R bodies are (i) EDTA and EGTA, (ii) temperature, and (iii) urea concentration (9, 31). Gibson et al. (31) reported that type Pa R bodies unroll when treated for only 15 min with 0.1 M EDTA or EGTA and that similar results are seen for type 51 R bodies. They also stated that the unwinding of type Pa R bodies is promoted by incubation at 50°C for 1 h and that increased temperature promotes unrolling of type 51 R bodies. Finally, they observed that type Pa R bodies unroll more readily in the presence of urea. They also reported that type Pt and type 51 R bodies respond to urea in the same manner as type Pa R bodies. The effects of the above treatments on type Pa R bodies were observed to be irreversible.

Genetic determinants for R-body production in *P. avenae*. No bacteriophages or bacteriophagelike particles have been observed in the many sections of R-body-containing *P. avenae* cells that have been viewed by electron microscopy. Attempts to find plasmids have also proven negative (G. Willis and I. Gibson, unpublished data). We therefore assume that the genes for R-body synthesis are localized on the chromosome of *P. avenae*. The R-body-encoding DNA sequences of *P. avenae* do not possess detectable homology with those of *C. taeniospiralis* (32).

***P. avenae* R-body proteins.** Purified R bodies of *P. avenae* have been subjected to techniques that normally solubilize

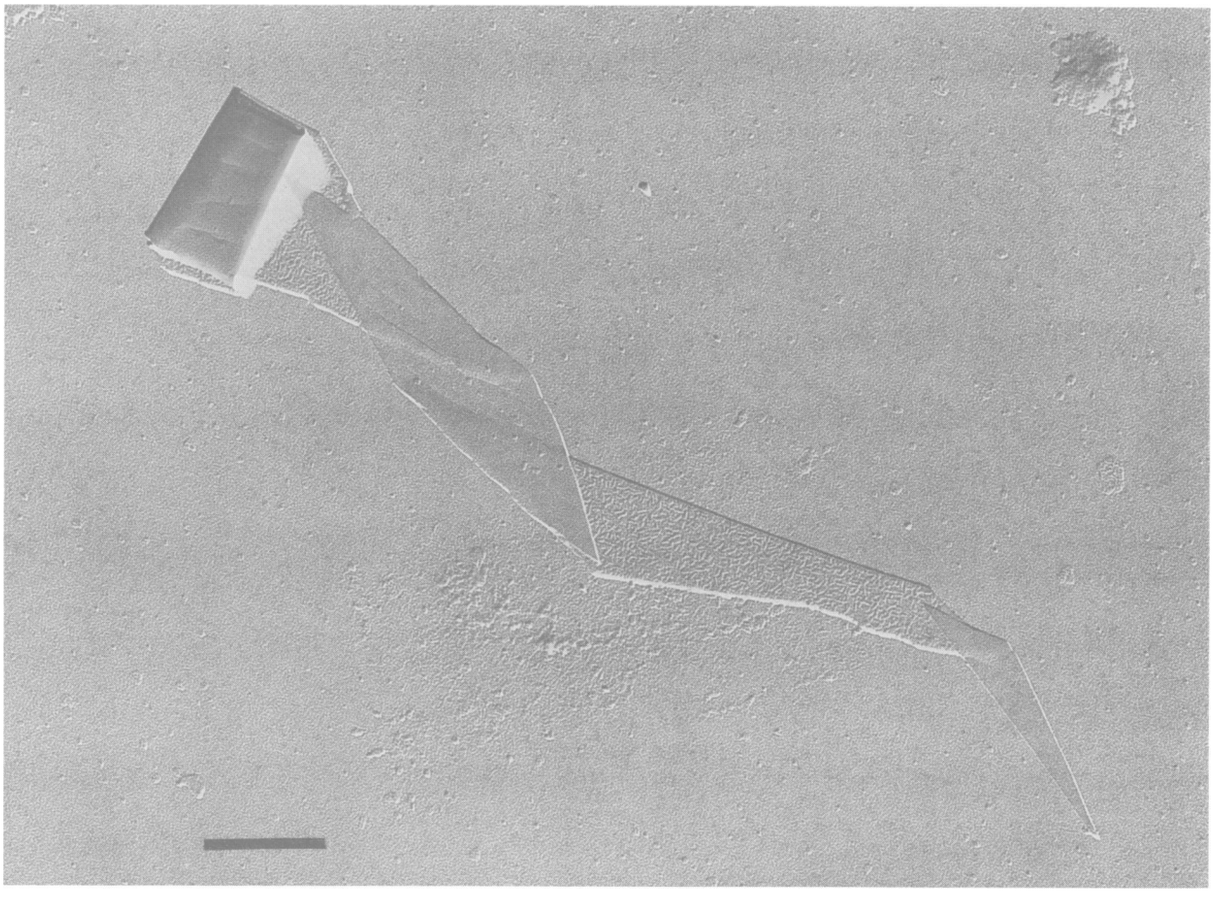


FIG. 25. Type Pa R body unrolling from the outside. Bar, 0.5 μm. Micrograph courtesy of B. Wells.

proteins (e.g., 8 M urea, SDS, 2-mercaptoethanol, and boiling), and the extracts have been electrophoresed on SDS-polyacrylamide gels (32). These R bodies are very resistant to such treatments. Complete solubilization does not occur, since R bodies can still be isolated from the tops of the gels. Three major protein bands (26, 34, and 50 kDa) are seen following these treatments, as well as minor bands at higher molecular masses. Further work is necessary to determine whether these are polymers of smaller units as described for *C. taeniospiralis*.

Toxicity of *P. avenae* R bodies. Gibson (unpublished data) has separated R-body-containing *P. avenae* cells from those without R bodies and inoculated leaves of oats, barley, corn, and rye. Characteristic pathogenic symptoms were induced in all plants by R-body-containing bacteria but not by R-body-free bacteria. In addition, Gibson (unpublished data) has isolated from 7-day-old *P. avenae* cultures, using fast protein liquid chromatography, a toxic protein causing the characteristic symptoms in maize plants. Although inducing necrosis at concentrations of 0.1 fg/ml, it did not cause the death of axenically grown paramecia after 3 days. The toxin has been termed avenaein. A mutant of *P. avenae* which does not produce R bodies fails to produce levels of avenaein detectable by fast protein liquid chromatography methods or by biological activity against maize plants. At present, molecular weight estimations of the protein are 5,500 to 7,000, and studies are under way to determine at what level the plant cells are affected by the bacteria, e.g., extracellularly or intracellularly. This is the first report that a toxin from an R-body-containing bacterium has been isolated, and this toxin is apparently independent of the actual R-body structure. Sequencing studies should make possible the use of molecular methods to isolate the toxic genes and to determine the role of the R body in the development of the toxic phenotype. Gibson speculated that toxin and R bodies may be related by a common biochemical pathway; for example, toxin may be a by-product in the production of R bodies or vice versa. He also reported that the toxin does not appear to destroy plant cells but may cause ion imbalances or leakage of ions. However, the mechanism of toxic activity and the role of R bodies in this process remain uncertain.

Strain EPS-5028

The R-body-producing bacterium strain EPS-5028, isolated from soil collected near Barcelona, Spain, was studied initially for its production of extracellular polysaccharide (16). This is a gram-negative organism with two polar flagella and with the ability to grow in the absence of combined nitrogen (26). EPS-5028 cells can utilize a variety of sugars and organic acids as their sole carbon source.

Strain EPS-5028 is believed to be a pseudomonad, although it does not appear to be closely related to any known *Pseudomonas* species (16, 26). Fusté et al. (26) compared strain EPS-5028 with the other known free-living, R-body-producing bacteria, *P. taeniospiralis* and *P. avenae*. The G+C content of EPS-5028 DNA was 65.1 mol% (26). This value is intermediate between the G+C contents of *P.*

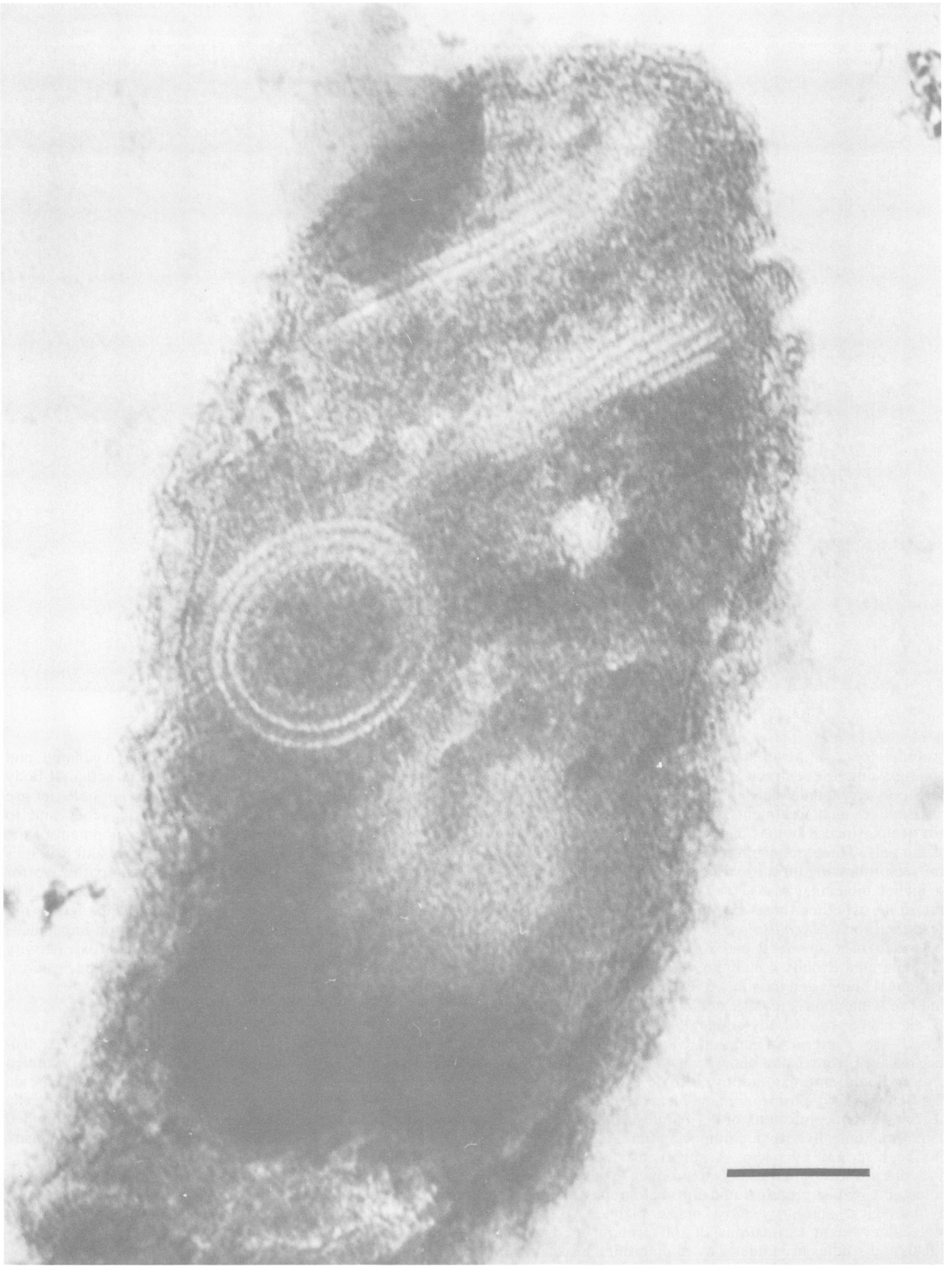


FIG. 26. Thin section of *P. avenae* showing two type Pa R bodies present in single cell. Bar, 1.0 μm. Reprinted from reference 11 with permission of *Micron and Microscopica Acta*.

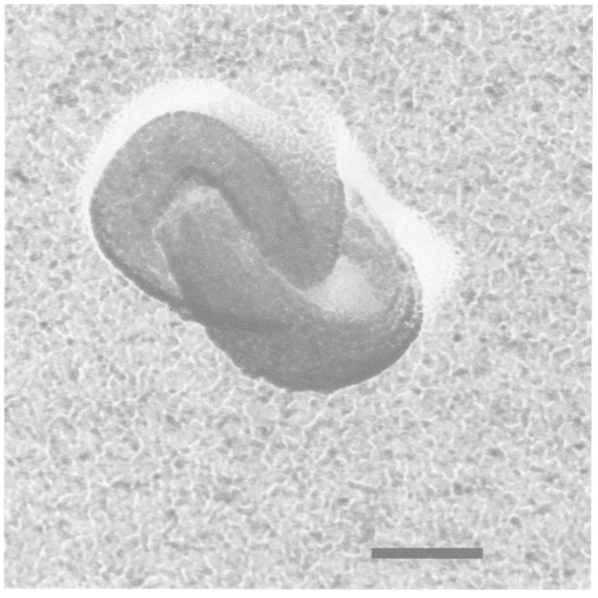


FIG. 27. Two interlinked type Pa R bodies. Stereoviewing angle with respect to normal, 45°. Bar, 0.5 μm. Micrograph courtesy of B. Wells.

taeniospiralis (56) and *P. avenae* (18, 93) DNA (60.4 and about 70 mol%, respectively). These three R-body-producing bacteria differ in a number of physiological characteristics as well (26).

Fusté et al. (26) reported the presence of R bodies in EPS-5028. R bodies were found in EPS-5028 cells harvested from cultures in different growth phases. Detailed measurements have not been made on these R bodies, but they appear to be slightly smaller than *P. taeniospiralis* R bodies in all respects. They may constitute yet another R-body class. Fuste et al. (26) also showed that after ultraviolet irradiation of EPS-5028, bacteriophages and structures resembling bacteriophage heads were visible. These structures were not seen in cells treated with the antibiotic mitomycin C. As is the case for the defective prophages described by Lalucat et al. (55) in *P. taeniospiralis*, there is no direct evidence to link the R bodies found in EPS-5028 with a bacteriophage genome. The coincidence of R bodies and prophages being simultaneously present is, however, intriguing. Also interesting is the fact that both EPS-5028 and *P. taeniospiralis* were isolated from soil collected in the vicinity of Barcelona, Spain.

RELATIONSHIPS AMONG R-BODY CLASSES

Five classes of R bodies have been described. Type 51, type 7, and type Cc R bodies are found in different *Caedibacter* species. *P. avenae* R bodies (type Pa) and *P. taeniospiralis* R bodies (type Pt) constitute two additional classes. All of these structures share certain features. They are highly insoluable protein ribbons, typically coiled into cylindrical structures but capable of unwinding. Although this warrants the generic name R body, these structures differ in morphology and behavior. If these classes of R bodies are divergent forms, evolved from a common ancestral protein structure, homology should be detected (the extent of homology generally reflecting how recently structures have diverged).

Similarities in the Organization of R Bodies

R bodies representing type 51, type 7, and type Cc R-body classes have been analyzed by SDS-polyacrylamide gel electrophoresis (47, 48, 96). A common feature of the polypeptide patterns generated seems to be a ladderlike series of polymerization intermediates. More is known about the organization of type 51 R bodies than about other classes of R bodies because the genetic determinants for type 51 R bodies have been cloned and expressed in *E. coli*, from which large amounts of type 51 R bodies may be obtained. Subjecting purified type 51 R bodies to SDS-polyacrylamide gel electrophoresis results in a specific pattern of polypeptides with molecular masses ranging from about 10 kDa to masses too high for the polypeptides to enter the stacking gel, with a ladderlike series of polypeptides extending from about 40 kDa into the stacking gel (48). The ladderlike pattern, believed to represent polymerization intermediates, has been observed in the electrophoretic patterns generated for type 7, Cc, and Pt R bodies (48, 68, 96).

The ladderlike polypeptide patterns for other R-body types are seen more readily in immunoblots because of the sensitivity of this technique. R bodies electrophoresed on SDS-polyacrylamide gels and then blotted to nitrocellulose filter paper, reacted against homologous R-body antiserum, and developed by exposure to horseradish peroxidase-conjugated anti-rabbit immunoglobulin G and the appropriate substrate revealed identical polypeptide patterns as seen in the polyacrylamide gels. Thus, immunoblots confirm the polypeptide patterns described above.

Although the polypeptide patterns generated by SDS-polyacrylamide gel electrophoresis of different R bodies have a similar ladderlike appearance, they differ in the size and number of low-molecular-mass polypeptides (presumably structural subunits and R-body-associated enzymes) and the size and extent of polymerization intermediates. These differences suggest that R-body types are divergent at the molecular level. The presence of a ladderlike polypeptide series in different R-body classes suggests that, like type 51 R bodies, R-body assembly utilizes a polymerization process involving covalent bonds other than disulfide bridges.

Serological Relationships among R Bodies

Serological techniques have been used to evaluate how closely related are the different R body classes. Kappa R bodies appear to be related to one another (68), but pseudomonad R bodies show little, if any, cross-reactivity to one another or to the classes of kappa R bodies (32, 47, 57). However, it has been difficult to distinguish weak or limited cross-reactivity from nonspecific binding or binding to a cross-reactive contaminant.

Rabbit antiserum raised against type 51 R bodies has been reacted against Western immunoblots of SDS-polyacrylamide gels of R bodies from the different classes. The antiserum was raised against R bodies synthesized in *E. coli* that had been transformed by a recombinant plasmid carrying the R-body-encoding DNA sequence of *C. taeniospiralis* 47 (47). The antiserum reacted strongly with type 51 R bodies collected from different *C. taeniospiralis* strains. Anti-type 51 R-body serum has not been reacted against any type 7 R bodies, but antisera to type Cc and to type 7 R bodies (from both *C. varicaedens* 7 and *C. varicaedens* 562) have been tested against type 51 R bodies (68). Weak cross-reactions were observed with the ladderlike polypeptides in Western blots. However, cross-reactivity has not

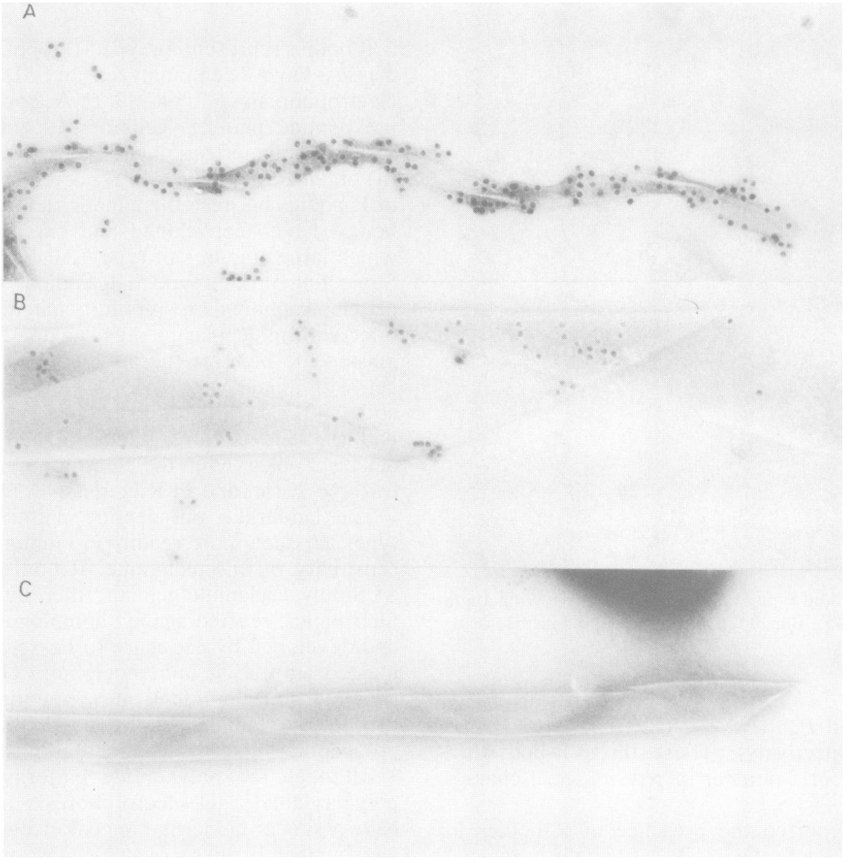


FIG. 28. Immunogold negative staining of R bodies from (A) *P. taeniospiralis*, (B) *P. avenae*, and (C) *C. taeniospiralis* by using antisera prepared against *P. taeniospiralis* R bodies (type Pt). Modified and reproduced from reference 57 with permission of *Micron and Microscopica Acta*.

been observed between antiserum to type 51 R bodies and either of the pseudomonad R bodies. Weak cross-reactivity may have occurred between antisera to type 51 R bodies and type Pt R bodies (47), but the researchers had reservations because the full polypeptide pattern did not react. They were therefore uncertain whether true but limited cross-reactivity was observed, or simply a nonspecific binding to a common contaminant.

In addition to Western blot assays, immunogold labeling has been used to assess R-body homologies. Type Pa R bodies were exposed to rabbit anti-type Pt R-body serum, reacted with anti-rabbit immunoglobulin G antibodies conjugated to colloidal gold, and then viewed under an electron microscope (57). Light labeling in the heterologous reaction with type Pa R bodies was observed, whereas reactions of this antiserum with its homologous antigen resulted in clear and specific labeling (Fig. 28). Apparently, cross-reactivity between the pseudomonad R bodies is present but weak. Gibson et al. (32), using antisera prepared against type 51 R bodies, were unable to detect any cross-reactivity between type Pa R bodies and type 51 R bodies by immunogold labeling (Fig. 29).

The immunogold-labeling technique has also been applied in studies with other R body types. Antisera against the three types of *Caedibacter* R bodies (types 51, 7, and Cc) were tested against type 51 R bodies (68). Cross-reactivity was clearly evident (Fig. 30). The accumulation of such data points to the conclusion that, although the different R-body classes are inherently different, there is evolutionary homology among *Caedibacter* R bodies. The relationships between the two *Pseudomonas* R bodies and between *Pseudomonas* and *Caedibacter* R bodies are less certain.

Relationships among R-Body-Coding DNA Sequences

R-body production and toxigenicity in *Caedibacter* species is correlated with the amplification of extrachromosomal elements (19, 90). If, as present evidence indicates, extrachromosomal DNA directs the expression of these traits, there should be homology among the extrachromosomal elements found in the various kappa species. DNA-DNA hybridization and analysis of the patterns generated by agarose gel electrophoresis of DNA fragments obtained following restriction endonuclease digestion have been used as a means for assessing relationships among extrachromosomal elements.

All *C. taeniospiralis* strains contain closely related R-body-encoding plasmids. Physical maps have been generated for plasmids from eight strains by restriction endonuclease mapping (Fig. 16). Excluding the transposons that are inserted at different positions in the various plasmids, the physical maps are identical (86, 91). The R-body-encoding regions of pKAP47 and pKAP116 have been sequenced, and no difference between the two sequences were detected

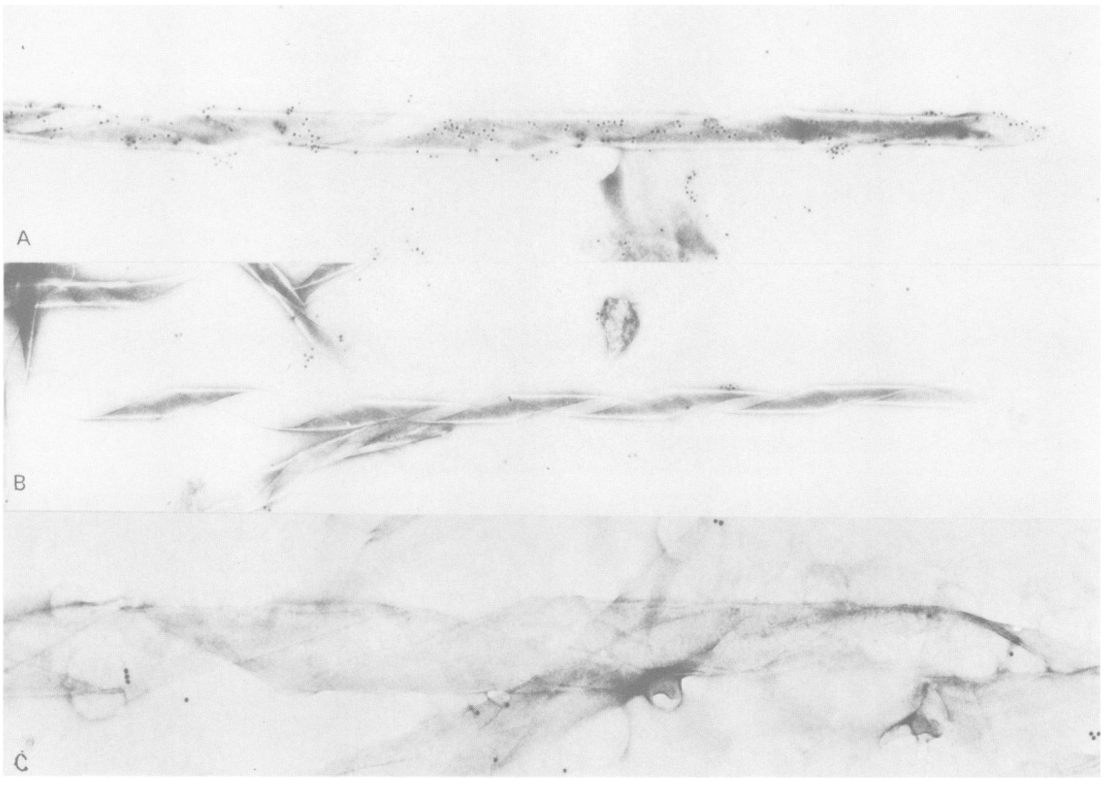


FIG. 29. Immunogold negative staining of R bodies from (A) *C. taeniospiralis*, (B) *P. taeniospiralis*, and (C) *P. avenae* with antisera prepared against *C. taeniospiralis* R bodies (type 51). Micrographs courtesy of B. Wells, R. Quackenbush, and I. Gibson. Modified and reproduced from reference 31 with permission of *Micron and Microscopica Acta*.

(Heruth, Ph.D. thesis). Figure 19 compares restriction maps for these R-body-encoding regions and again shows the high degree of homology that exists among these plasmids. These observations agree with the results of hybridization studies (90), in which a DNA probe generated from the pKAP47 R-body-encoding sequence hybridized with DNA from other *C. taeniospiralis* strains. These observations are further supported by serological studies (47), which show cross-reactivity between type 51 R bodies from various *C. taeniospiralis* strains (see above). The similarity of *C. taeniospiralis* plasmids are quite remarkable, considering the geographic distribution of the host paramecia from which they were collected.

Analysis of extrachromosomal elements in other kappa species has revealed that at least three different bacteriophage genomes contain R-body-encoding sequences, and homology has not been detected between these sequences and *C. taeniospiralis* plasmids (90). DNA purified from the bacteriophage Cv562 showed no significant homology, assessed by DNA-DNA hybridization, with plasmid DNA from *C. taeniospiralis* 51 or with total cell DNA from *C. varicaedens* 511 (kappa strains 562 and 511 belong to the same subspecies) (85).

Less than 2,000 bp is required to direct type 51 R-body synthesis (48; Heruth, Ph.D. thesis). It is therefore possible that the extrachromosomal DNA sequences coding for R-body production share homology which is not detected by the methods described above. To test for this, Quackenbush et al. (90) generated a probe from the R-body-encoding DNA sequence of pKAP47 by nick translation and attempted to hybridize this sequence with Southern blots of total cell DNA collected from various kappa strains. All *C. taeniospiralis* strains hybridized strongly with this probe. This was expected from the high degree of homology seen in the physical maps of the R-body-encoding plasmids. Representatives of the three *C. varicaedens* subspecies (strains 7, 511 and 562, and 1038) and *C. pseudomutans* 51m1 were tested. Only strain 1038 was able to hybridize detectably with the probe. This is an interesting result since Preer et al. (80) noted that strain 1038, like *C. taeniospiralis*, does not contain spherical bacteriophages but instead produces helical structures that are possibly aberrant products of capsid assembly. Quackenbush et al. (90) suggested that the genetic determinants for *C. varicaedens* 1038 may be intermediate between the sequences coding for type 51 and type 7 R bodies and that kappa R bodies, whether plasmid or bacteriophage encoded, have a common origin. This probe (generated from type 51 R-body-encoding sequences) also failed to hybridize to *C. caryophila* total cell DNA (94).

The probe DNA generated from the pKAP47 R-body-encoding DNA sequence has also been reacted against Southern blots of *P. taeniospiralis* DNA, but it failed to hybridize (47). The dissimilarity between R-body-encoding regions and extrachromosomal DNA of the different kappa strains and *P. taeniospiralis* DNA demonstrates that type 51 R bodies are only distantly related to type 7 or type Pt R bodies.

The evidence presented above demonstrates that the sequences coding for the various R-body types are distinct from one another. The serological data indicate that there is some relationship between the caedibacterial R-body types

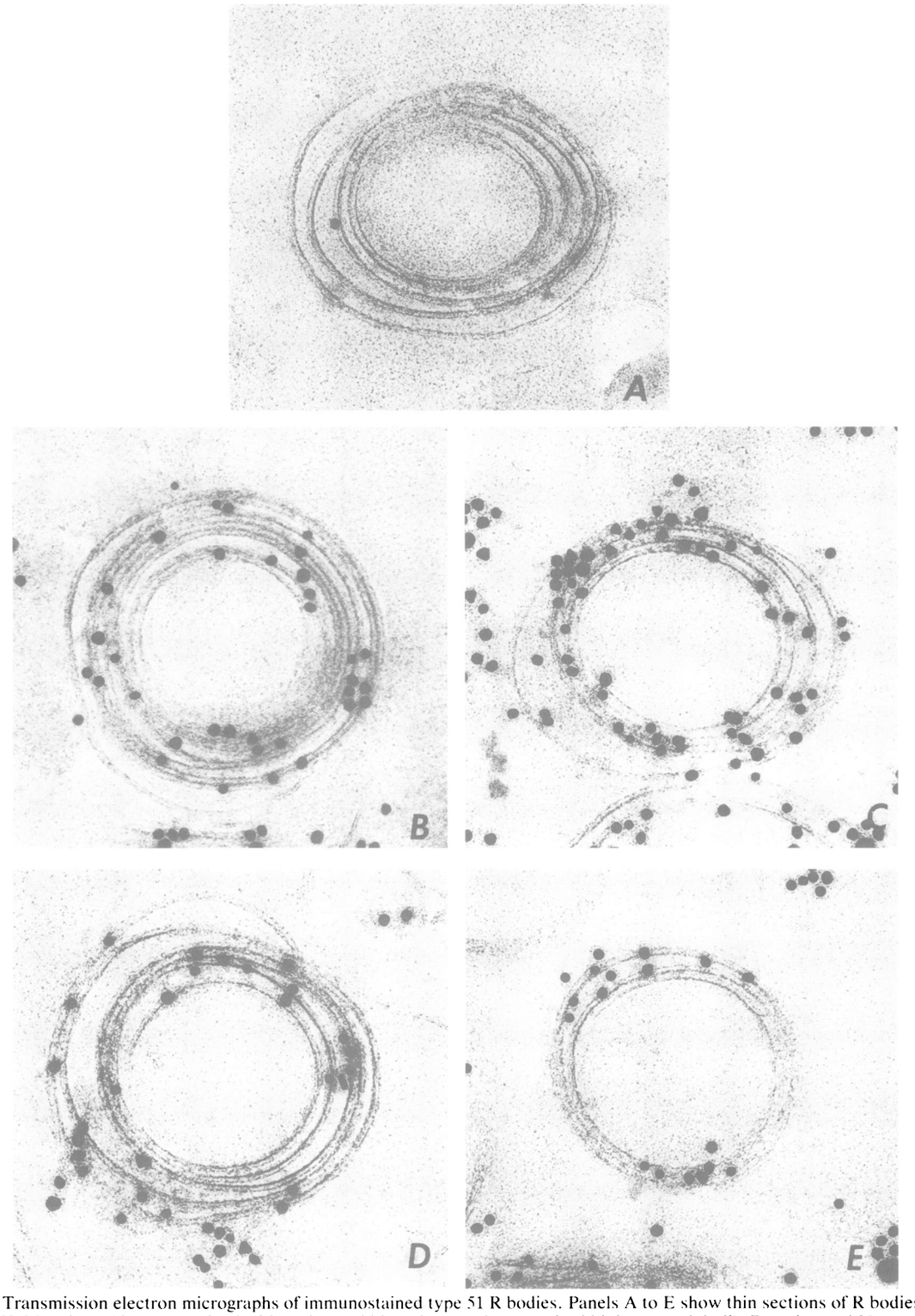


FIG. 30. Transmission electron micrographs of immunostained type 51 R bodies. Panels A to E show thin sections of R bodies embedded in Lowicryl K4M resin and immunostained with various rabbit antisera and goat anti-rabbit immunoglobulin G-conjugated 20-nm gold colloid. The antisera used in staining are as follows: (A) anti-saline serum as a control; (B) anti-type 51 R-body serum; (C) anti-type 7 R-body serum (*C. varicaedens* 7 R bodies); (D) anti-type 7 R-body serum (*C. varicaedens* 562 R bodies); (E) anti-type Cc R-body serum. Magnification, ×54,000. Reprinted from reference 68 with permission of *Micron and Microscopica Acta*.

and further indicates the uniqueness of each of the R bodies produced by the pseudomonads.

EVOLUTION OF R BODIES

Evolution of *Caedibacter* R Bodies

There are several puzzling aspects about the evolution of R-body-containing endosymbionts. There exists an interaction among host, endosymbiont, and extrachromosomal DNA that must be taken into account, and although divergence is seen in each of these genetic systems, there are also some highly conserved features in kappa-*Paramecium* symbioses. Furthermore, closely related kappa strains are found on different continents.

Evolution of kappa endosymbionts. The evolution of these symbioses and the extent of divergence and conservation are of particular interest and should be pursued, because the kappa-*Paramecium* relationship may be comparable to hypothesized endosymbioses which resulted in the evolution of eucaryotic organelles (e.g., mitochondria and chloroplasts) (61). The scenario advanced by Margulis (61) concerning the origins of eucaryotic organelles is that, after symbiosis was established, metabolic and genetic integration occurred. Presumably this was a slow process during which the symbiont lost its autonomy, becoming dependent upon its host for certain metabolic functions. Endosymbionts in *Paramecium* species exhibit varying degrees of autonomy. The symbionts mu (128) and lambda (123, 128) have reportedly been cultured outside their hosts in axenic media, although their growth is extremely poor. Kappa particles have not been cultured outside their hosts, despite much effort to do so, and are therefore considered obligate endosymbionts, dependent on *Paramecium*-encoded products for their survival (76). Thus, it has been suggested that the kappa-*Paramecium* relationships are evolving, as did the relationships that produced eucaryotic organelles. The kappa-*Paramecium* relationships are indeed suggestive of these earlier symbioses.

Host distribution. Consider the evolutionary history of paramecia. Excluding from consideration for the moment *P. caudatum*, the paramecia that maintain kappa particles belong to two sibling species in the *P. aurelia* complex. These species, *P. biaurelia* and *P. tetraurelia*, are strictly confined to freshwater environments, but their distribution is worldwide. Populations on different continents are presumed to be completely isolated from one another, since there are no obvious ways that paramecia can transcend saltwater barriers. It has been reported that paramecia are transported from one water system to another in the wet fur of mammals (60). While such transport, in conjunction with heavy rains and flooding, may allow movement between separate but adjacent water systems on a given land mass, it is hardly sufficient to break down the isolation of populations separated on different continents.

Were a cyst present in the life cycle of a paramecium, these barriers might be easily conquered. There are unconfirmed reports of encystment in various paramecia, including *P. aurelia* (62). However, paramecia have been studied extensively under a variety of conditions, and the consensus among protozoologists is that paramecia do not form cysts (126). Consequently, we must assume that gene flow between these geographically isolated populations is nonexistent.

It has been suggested (71a, 85, 126) that paramecia may be transported by human activity. For example, if water containers are filled in one river system and then carried some distance, perhaps even across oceans, where they are emptied and refilled in another river system, it is possible that paramecia could be transported considerable distances, from one water system to another. It is possible that human activity has thus inadvertently inoculated river systems around the world with particular strains of paramecia.

Kappa-*Paramecium* symbiosis may be ancient. Paramecia containing kappa particles have been collected from locations around the world. If the host paramecia on different continents are isolated from one another, how can we account for the presence of particular kappa species on different continents? One possibility is that the symbiotic relationships between kappa and paramecia were established prior to separation of the land masses. This possibility has been raised previously by Quackenbush (85). If kappa-bearing paramecia were established prior to onset of continental drift, we could explain the presence of kappa particles in widely separated locations such as Australia and North America. However, this solution to the evolutionary puzzle is not without problems. The most widely distributed kappa species, *C. taeniospiralis* (78), possesses highly conserved extrachromosomal elements. Plasmids isolated from *C. taeniospiralis* populations collected in Japan, Australia, North America, and Panama are nearly identical (86). Is it likely that these species could have been isolated for tens of millions of years and countless generations without substantial divergence in these DNA sequences having occurred? This does not seem to be a likely scenario.

Distribution of some kappa-containing paramecia may be recent. If one grants that humans have inadvertently been responsible for distributing paramecia to new locations, we need not invoke elaborate explanations about why kappa species on different continents carry identical or nearly identical extrachromosomal elements. However, we do not have enough data concerning the relationships of kappa-bearing paramecia and kappa-free paramecia in various locations to evaluate whether the kappa-bearing paramecia represent introduced varieties. This explanation would gain credence, if, for example, the *P. tetraurelia* populations in Australia and Japan which harbor *C. taeniospiralis* symbionts were shown to be distinct from other Australian and Japanese *P. tetraurelia* populations but closely related to *C. taeniospiralis*-containing *Paramecium* strains found in North America. At present, we can only speculate, since not enough is known about *Paramecium* relationships to decide this question.

R-body evolution in distantly related kappa species. As implied earlier, the assumptions that R-body-producing kappa symbionts have a common origin and that R-body evolution parallels kappa evolution present difficulties in explaining kappa distribution. However, if we discard the idea that the evolution of R bodies and kappa are necessarily related, we may find a more productive line of thought. Furthermore, considering that R bodies are encoded by extrachromosomal elements, it is quite plausible that kappa and R-body evolution are divorced from one another. Let us consider, then, the argument that R bodies, but not necessarily their kappa hosts, have a common origin.

DNA-DNA hybridizations indicate that kappa species are genetically quite distinct from one another (83). If these species have a common heritage, did divergence occur before or after the establishment of symbiosis? If extensive divergence occurred in a line of R-body-containing, free-living bacteria, is it likely that several distinct branches would establish, independently of one another, similar sym-

biotic relationships with paramecia? On the other hand, when a bacterium colonizes a rich and unexploited environment such as the cytoplasm of a paramecium, it is plausible that a period of rapid divergence would ensue, since symbiotic balance presumably can be established by various strategies. Although such a hypothesis appears to provide a more reasonable explanation for the diversity of R-body-producing symbionts in *Paramecium* species, it demands the establishment of symbiosis and divergence of symbionts prior to separation of the continents. Even granting the distribution of some kappa strains by human intervention, the distribution of kappa species is not reasonably explained in this way. Thus, considering the divergence of kappa species, the apparent conservation of genetic elements, the similarity of symbiotic relationships among different species of kappa and paramecia, and the distribution of kappa species, it is difficult, in light of the current understanding of evolutionary processes, to construct a reasonable explanation of R-body and kappa evolution from the assumption that the two evolutionary histories are closely tied to one another.

It is possible that the evolutionary origins of R bodies and kappa particles are separate, and the kappa symbionts themselves may be quite distinct. Since R bodies are encoded by extrachromosomal elements, and DNA-DNA homology is very low among kappa species, it is possible that the genus *Caedibacter* is an artificial grouping, reflecting only the fact that these organisms have acquired extrachromosomal elements that carry related genes for R-body synthesis. One need only postulate that the bacteria, as symbionts or free-living bacteria, were exposed to and able to acquire such extrachromosomal elements (possibly these genetic elements were transferred from one symbiont to another in a coinfected paramecium). This suggestion reflects the observation that DNA-DNA homology between *C. pseudomutans* 51m1 and *C. varicaedens* 562 is less than 15%, but homology between their bacteriophages (which are believed to encode R-body production) was reported to be 73% (90). It should be noted, however, that not all extrachromosomal elements in kappa particles are closely related (83, 90, 91).

Although there is no direct evidence to support this hypothesis, such an explanation is credible in light of recent work showing that *Chlorella*-like symbionts of *P. bursaria* are infected by a large, double-stranded DNA virus. Surprisingly, it was shown that a variety of double-stranded DNA viruses, collected from fresh water throughout the United States, are able to infect a *Chlorella* isolate from *P. bursaria* (116, 117). Such viruses appear to be common and have been found in concentrations up to 4×10^4 plaque-forming units/ml (96, 120). Furthermore, apparently identical viruses have been collected from widely separated locations (118).

Chlorella-like algae also exist in symbiosis with *Hydra viridis* (64, 119). Virus particles are not seen in these symbionts when they are within the hydra, nor are viruses seen in the hydra itself. However, after algae are isolated from their host, viruses appear in the algae within 3 to 6 h, and the entire population of isolated algae is lysed within 24 h. Presumably, the viruses are lysogenic in the algae and enter the lytic phase once the algae are free of their hydran host (121). The unexpected discoveries of a reservoir of large double-stranded DNA viruses in freshwater environments, the ability of these viruses to infect a *Chlorella*-like alga, and the presence of similar lysogenic viruses in the *Chlorella*-like endosymbionts of *H. viridis* and *P. bursaria* are sufficient to consider seriously the hypothesis that R-body-encoding extrachromosomal elements have been acquired by otherwise distantly related or even unrelated bacterial *Paramecium* symbionts. However, such an explanation is complicated by the apparent defectiveness of the R-body-encoding bacteriophages.

Summary. Explaining the evolution of kappa species is tied in part to understanding the distribution of kappa-*Paramecium* symbioses. If we assume that kappa-bearing paramecia were distributed prior to separation of the continents, kappa are unusual in the extent to which certain elements have been conserved. If we assume that human activities have played a role in paramecium transport, the distribution of kappa species may not in all cases be pertinent to discussions of divergence and conservation of genetic elements.

That caedibacteria are obligate endosymbionts suggests that the symbiotic relationships under consideration are of ancient origin. Paramecia have probably existed for over 2×10^8 years (see discussion in reference 126). Since their appearance, these organisms have undoubtedly been subjected to bacterial invasions. Some colonizing bacteria were benign (at least under some conditions), eventually becoming established endosymbionts. In regard to the R-body-producing endosymbionts (caedibacteria), we suggest that (i) several symbiotic relationships involving different bacterial and *Paramecium* species were established independently; (ii) these bacterial endosymbionts share related R-body-encoding extrachromosomal elements; and (iii) although an initial segregation of some *Paramecium-Caedibacter* systems may have occurred prior to, or resulted from, separation of the continental land masses, the worldwide distribution of some caedibacterium-containing *Paramecium* species (notably the *P. tetraurelia-C. taeniospiralis* system) may be recent. Consequently, the evolutionary history of these symbioses may be independent and distinct in some respects, while possessing common hosts and related extrachromosomal elements.

Relationship between *Caedibacter* and *Pseudomonas* R Bodies

Do *Caedibacter* and *Pseudomonas* R bodies have a common ancestry, and, if so, can R bodies be used as phylogenetic markers (i.e., does their presence in these species indicate a close relationship between these genera)? Three possibilities come to mind when considering these questions: (i) *Caedibacter* and *Pseudomonas* species have a common lineage, and R bodies are a primitive characteristic retained in a few modern species; (ii) these genera are not closely related, but R-body genes have a common lineage (possibly transmitted between these genera by extrachromosomal elements); and (iii) the R bodies are products of covergent evolutionary events evolving independently in different bacterial lineages.

R bodies are unique protein structures, found in only a few bacterial species. Therefore, a reasonable assumption is that the species possessing these novel structures are closely related. Although the R-body-containing species that have been described may be evolved from a common R-body-containing ancestor, the relationship among all these species cannot be described as close. At least three bacterial genera are represented by R-body-containing species: *Pseudomonas*, *Caedibacter*, and *Rhodospirillum*. The known characteristics of *Caedibacter* species indicate a remoteness from *Pseudomonas* species. The G+C content measures 67 to 71% in *P. avenae* (18, 94), 60.4% in *P. taeniospiralis* (56),

65.1% in strain EPS-5028 (26), but only 40 to 44% in *Caedibacter* species (85). Because *Caedibacter* species cannot be cultured outside their hosts, it has been difficult to obtain adequate data to establish taxonomic relationships. *Caedibacter* species are dissimilar from known free-living bacteria, probably as a result of their evolution as obligate endosymbionts. The consequence of divergence from their postulated free-living ancestors is that relationships with established bacterial groups have not been determined. However, it is highly unlikely that *Pseudomonas* and *Caedibacter* are closely related genera. An investigation involving molecular markers of evolutionary relationships, for example, 16S RNA analysis, may prove valuable in addressing such questions.

Regardless of the relationship between these bacteria, is there enough evidence to argue for a phylogenetic relationship among the various R-body types? It is possible that an evolutionary relationship among R bodies is not dependent upon, and need not follow or even parallel, the relationships of the bacteria possessing them. Quackenbush (85) suggested that, because *Caedibacter* R bodies are encoded by extrachromosomal elements, this genus may be a collection of phylogenetically distant bacteria that have acquired related extrachromosomal DNA (see above). However, there is no strong evidence to suggest that *Pseudomonas* R bodies are encoded by extrachromosomal DNA. However, it is not inconceivable that R-body-encoding genes have been distributed to various bacterial species by extrachromosomal elements.

Finally, we consider the possibility that R bodies evolved independently in at least two genera, *Pseudomonas* and *Caedibacter*. As discussed above, there is very little, if any, protein homology among R bodies from *P. avenae*, *P. taeniospiralis*, and *C. taeniospiralis* (as determined by serological techniques). However, the absence of strong immunological cross-reactivities does not preclude an evolutionary relationship. Independent evolution of coiled protein ribbons, capable of unrolling, is not inconceivable, yet all R bodies may share similarities in their construction. The available evidence, discussed here, strongly indicates that type 51 R bodies are composed of covalently linked polypeptide polymers involving a single major subunit and further indicates that this may be the case for the other R-body types, including *P. taeniospiralis* and possibly *P. avenae* R bodies. The unwinding behavior and association with killing traits provide further evidence, albeit circumstantial, that these are related structures. Thus, a hypothesis that all R bodies are distantly related proteinaceous structures with a common origin cannot be ruled out.

Relationship of R Bodies and Ejectisomes

Ejectisomes, the extrusive organelles found in members of the families *Cryptophyceae* and *Prasinophyceae*, have been compared to R bodies (37). Ejectisomes are cylindrically coiled ribbons enclosed in a single membrane, and they unroll to form hollow tubes. Cryptophyte and prasinophyte ejectisomes share a superficial similarity in morphology and behavior which has been cited as evidence for a close phylogenetic relationship between these algal groups (116, 117). However, although they appear similar in coiled and uncoiled states, there are substantial differences between these ejectisome structures. Electron-microscopic analysis has revealed that the size, shape, and arrangement of ejectisome substructures differs between ejectisomes from *Cryptomonas* 58 and *Pyramimonas parkeae*, leading Morrall and Greenwood (65) to question the relatedness of cryptophyte and prasinophyte ejectisomes. *Pyramimonas* ejectisomes unroll in a telescopic fashion, and the spirals of the extended ribbon overlap to form a hollow tube (33). Cryptophyte ejectisomes, although once assumed to unroll in this manner (41), are now believed to unroll from the outside (37, 42, 65). Cryptophyte ejectisomes are also quite different in other respects. They are bipartite, consisting of interconnected major and minor coiled elements (2). In the coiled resting state, the minor element is nested in a funnel-shaped depression at one end of the major coil. The ribbons that form the major and minor coiled elements are tapered and are connected at their widest ends. When extended, the ribbons curl along their longitudinal axes to form hollow tubes which are set at an angle to one another.

Although there are apparently two classes of ejectisomes, cryptophyte and prasinophyte ejectisomes, both structures are, at least superficially, comparable to R bodies, and the question remains of whether the similarities between ejectisomes and R bodies represent homology or whether these structures are independently evolved. The resemblance of kappa R bodies and ejectisomes was noted by Hovasse (41), and these structures were compared by Hovasse et al. (42) and Schuster (98, 99). In general, ejectisomes are rolled more tightly than R bodies, and there is greater size variation between different classes of R bodies than is seen among ejectisomes (42). Telescopic unrolling is found in *C. taeniospiralis*, *C. macronucleatum*, and *P. taeniospiralis* R bodies, and also in prasinophyte ejectisomes. All other *Caedibacter* R bodies, *P. avenae* R bodies, and cryptophyte ejectisomes unroll from the outside. The extended cryptophyte ejectisomes, but not extended R bodies, then curl lengthwise to form hollow tubes. The tapered ribbons of cryptophyte ejectisomes, joined at their widest ends, form a structure that is perhaps most similar to *P. avenae* R bodies, unrolled ribbons of which are distinguished morphologically by tapering outward from the middle to a point at each end (125).

Preer et al. (76) found the similarities between kappa R bodies and ejectisomes striking but did not speculate about possible relationships. As they explained, it is difficult to conceive of bacteriophage-encoded, bacterial inclusion bodies that are genetically linked with a eucaryotic structure. On the other hand, convergent evolution of such structures also seems rather remarkable, since it is hard to imagine environmental pressures and biophysical constraints that would promote these similarities, especially considering the uniqueness of such structures. Questions concerning the origins of these structures are indeed interesting, but too little data are available to attempt to provide any answers. Reliance on superficial resemblance should be avoided in determining questions of phylogeny. It is possible that a more complete understanding of ejectisome and R-body structure will reveal that the resemblances are superficial and reflect convergence of form rather than true homology. Certainly, the divergence of the organisms involved makes this the most plausible explanation. Nevertheless, until we acquire a better understanding of ejectisome and R-body construction and behavior, we cannot eliminate the possibility that these structures are evolutionary progeny of an ancestral structure and diverged from one another in the distant past. Since the time that Preer et al. (76) commented on the resemblance of kappa R bodies and ejectisomes, the R-body-producing, free-living bacteria have been discovered, adding a new dimension to consider. The *Pseudomonas* R bodies, although quite similar to kappa R bodies, are, however, definitely different, representing two new R-body

classes. If we grant that *Pseudomonas* and *Caedibacter* R bodies may be phylogenetically related, we cannot eliminate a possible relationship between ejectisomes and R bodies. On the other hand, the existence of R-body-like structures in flagellates demands that the possibility of convergent evolution not be ruled out.

Relationship of R Bodies and Scroll-Like Membrane Systems

In addition to ejectisomes, another structure has been described which bears a resemblance to R bodies. Cyanobacteria are known to contain a wide variety of inclusion bodies (43). Among the variety of unusual inclusion structures are scroll-like membrane systems, found in *Gloeotrichia pisum* (44). These structures appear in electron micrographs to consist of a membrane ribbon coiled into a cylindrical form similar to R bodies. Jensen (43) suggested that these cyanobacterial inclusion bodies may be related to membranous spheres, found in *Nostoc linckia*, which appear to consist of two or three membrane sheets wrapped around a common center (44). Jensen (43) further noted the similarity of the scroll-like membrane system to the R bodies of *P. taeniospiralis* and kappa particles. Additional work has not been done regarding the scroll-like structures, and so it is uncertain whether these structures are similar to R bodies in any other respects, e.g., unrolling. Certainly, one must wonder, however, just how unique the R-body structure is after encountering such inclusions as those found in cyanobacteria and the ejectisomes.

CONCLUSIONS

The discovery of R bodies in free-living pseudomonads abolished the uniqueness of kappa particles with respect to these structures and raised the possibility that R-body-producing bacteria are more common than previously suspected. Since R bodies are often expressed at low frequencies, it is possible that some R-body-producing species have been overlooked and remain uncharacterized. Certainly, the discovery of three R-body-producing *Pseudomonas* species suggests that other R-body-producing pseudomonads may be found. Also, the presence of R bodies in *R. centenum* suggests that R bodies are indeed more widespread than previously suspected. Furthermore, it has long been recognized that ciliate endosymbionts are often lost from their host cells when the host cells are established in laboratory cultures. Might the number of *Paramecium* species containing R-body-producing endosymbionts be larger than currently thought? The two independent discoveries in recent years of macronuclear kappa particles in *P. caudatum* suggest that there may be additional R-body-producing endosymbionts awaiting discovery.

Kappa-bearing paramecia have been found in North America, Europe, Asia, and Australia. Data are not available for the South American and African continents. However, if kappa-bearing paramecia are indeed more widespread than current data show, it is likely that there are new kappa species to be found, which may provide an opportunity to piece together a more complete understanding of R-body and kappa relationships and, by extrapolation, may give us a fuller understanding of evolutionary processes.

The recent discoveries of R bodies in free-living bacteria and the presence of R-body-like structures in cyanobacteria and algae raise questions about the evolution of R bodies. Should the diverse nature of R-body-producing bacteria be interpreted to mean that various R-body types have separate evolutionary origins? If they have a common origin, the genetic relationships become very interesting, since kappa R bodies are encoded by either plasmid or bacteriophage DNA, whereas pseudomonad R bodies are apparently determined by chromosomal DNA (at least for *P. avenae* and *P. taeniospiralis*). Characterization of R-body-encoding genes from different organisms should furnish answers to these questions.

In addition to the puzzle of R-body evolution, other intriguing problems have been the focus of research during the past few years. These are questions about R-body expression, assembly, and function. It has long been known that R-body expression in kappa particles is tied to the nutrient status of the host cells. This also appears to be the case for R-body expression in pseudomonads. Work at the molecular level to describe the regulatory system for R-body gene expression in different R-body-producing species has begun and may shed new light on our understanding of gene regulation.

The R-body structure is unusual in its resistance to dissociation by techniques for protein solubilization and denaturation. Type 51 R-body proteins show odd behavior in SDS-polyacrylamide gels, migrating more slowly as the percent acrylamide increases. Type 51 R bodies are apparently assembled from a single structural subunit, and in the polymerization process these subunits are covalently linked. The biochemical basis for this covalent linkage is not yet known, nor has the unusual behavior of the R-body proteins in SDS-polyacrylamide gels been explained. However, the focus of research toward explaining the molecular processes leading to the assembly of these unusual protein structures and toward explaining the unusual character of the proteins themselves, will undoubtedly further our understanding of protein-protein interactions.

Furthermore, for many years, there have been questions regarding the relationship of kappa R bodies to the bacteriophage structures that are also found in these cells. Might R bodies represent an aberrant assembly of bacteriophage proteins? We are not yet close to unraveling the mysteries of R-body relationships and origins, but a step toward achieving this goal is to understand the nature of the R-body proteins and their assembly. Also, once cleaner antibody preparations or monoclonal antibodies have been obtained, we should be able to look more closely at protein relationships.

Finally, the question of toxicity and the role of R bodies in killing must be addressed. Understanding the mechanisms of killing and the involvement of R bodies in killing activities has eluded kappa researchers for over 40 years, but the cloning of R-body-encoding plasmids and the work under way to clone the R-body-encoding genes and toxin genes from different species may provide a means of approaching these problems. There are indications that membranes are the target site of all the R-body-associated toxins. Perhaps studies should focus on membrane differences in sensitive and resistant cells and on the interaction of R-body proteins with membranes. If toxicity is tied to perturbations in osmoregulation, as suggested by Jurand et al. (45), research into the killing activities may also be important to understanding membrane physiology and biochemistry.

At present, our understanding of R bodies is incomplete, and the important questions of their evolution and function, the regulation of their expression, and molecular aspects of their assembly have only begun to be answered. However, attention is being focused on these problems, and since genes for type 51 R bodies were cloned and expressed in *E.*

coli, studies over the past few years are allowing us to ask more detailed questions directed toward answering some of these questions that have plagued past researchers. These are not trivial questions, and the insight to be gained from an understanding of R bodies will certainly carry over into a variety of other biological systems as well.

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