Genetic Relationships among Bacterial Endosymbionts of *Paramecium aurelia*: Deoxyribonucleotide Sequence Relationships among Members of *Caedobacter*

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Genetic relationships among bacterial endosymbionts of *Paramecium* assigned to the genus *Caedobacter* Preer *et al.* 1974 were determined using DNA–DNA hybridization techniques. It was shown that mu, nu and pi endosymbionts are closely related to each other, but not to any of the strains of kappa endosymbionts tested, and that kappa consists of at least three distinct and genetically diverse groups. Therefore, it is proposed that *Caedobacter* be split into two genera, *Caedobacter* and *Pseudocaedobacter*, depending upon the ability or lack of ability, respectively, to produce cells containing R bodies. Strains were screened for covalently closed, circular DNA by ethidium bromide/CsCl centrifugation, but only pi and hump-killer kappa particles were found to contain this form of DNA.

INTRODUCTION

Various strains of the *Paramecium aurelia* species complex (Sonneborn, 1975) are infected by bacterial endosymbionts. Several types of these endosymbionts are notable because of their killing activity when exposed to sensitive strains of paramecia. Of the nine types of bacterial endosymbionts (J. R. Preer *et al.*, 1974) known to occur in the *P. aurelia* species complex, five have been assigned to a single genus, *Caedobacter*, containing four species:

1. *C. taeniospiralis*, commonly known as kappa and pi particles. Kappa particles are toxic to sensitive strains of paramecia. Each strain of kappa produces specific pre-lethal effects in affected paramecia. These are: (i) development of an aboral blister (hump-killing); (ii) immobilization (paralysis); (iii) development of an increasingly large vacuole or simple swelling (vacuolization); or (iv) reversal of the direction of rotation while swimming (spin-killing). Only certain individuals within a population of kappa particles, known as bright particles, can cause killing activity against sensitive paramecia (Smith, 1961; Mueller, 1963). These bright particles contain a large cylindrical refractile inclusion (R body), about 0.5 μm long and 0.5 μm diam., which is a tightly coiled proteinaceous ribbon about 10 μm long and 13 nm thick (Anderson *et al.*, 1964). Only kappa particles can produce R bodies. Pi particles were originally believed to be non-killer mutants of kappa which had lost the ability to produce R bodies.

2. *C. conjugatus*, commonly known as mu particles. Mu-bearing paramecia are referred to as mate-killers because cell-to-cell contact between killer and sensitive paramecia is required for killing to occur.

3. *C. falsus*, commonly known as nu particles. Nu particles cause no known killing activity against their host paramecia.

4. *C. minutus*, commonly known as gamma particles. Gamma particles cause a strong killing activity (vacuolization) against sensitive paramecia.

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Recent investigations (Dilts, 1977; Quackenbush, 1977*a, b*) have suggested that *Caedobacter* may consist of genetically diverse groups of bacteria. This communication describes attempts to determine to what extent representative strains of the major groups within *Caedobacter* are genetically related on the basis of DNA homologies. Special attention is given to the genetic relationships of five of the six classes of kappa particles described by Preer *et al.* (1972).

The genetic determinants of the distinguishing trait (R body production) of kappa particles are most probably carried on extrachromosomal elements within them (Preer *et al.*, 1971; Dilts, 1976, 1977). Since some of these extrachromosomal elements are known to be covalently closed, circular DNA (Dilts, 1976, 1977; Quackenbush, 1977*b*), an attempt was made to determine whether this form of DNA occurs generally among the members of *Caedobacter*.

METHODS

Organisms. The paramecia used (Table 1) included representatives of five of the six classes of kappa described by Preer *et al.* (1972). All strains were obtained from the culture collection of J. R. Preer, Jr (Department of Biology, Indiana University, Bloomington, Indiana, U.S.A.) except for those bearing 138 mu and 139 pi (Table 1) which were kindly provided by A. T. Soldo (Research Laboratories, Veterans Administration Hospital, Miami, Florida, U.S.A.). *Escherichia coli* B was obtained from the Microbiology Culture Collection, Indiana University.

Media and growth conditions. Paramecia containing 138 mu, 139 pi and 1010 nu were grown axenically as described by Soldo *et al.* (1966) at 27 °C. Those containing 51 kappa and 540 mu were grown using *Chlamydomonas reinhardtii* as the food organism at room temperature (20 to 25 °C) as described by L. B. Preer *et al.* (1974) and Quackenbush (1977*a*). All other strains were grown using *E. coli* B as the food organism in Cerophyl medium (Sonneborn, 1970); those containing class 51 kappas were grown at room temperature, those containing class 7 kappas, 511 kappa, 1038 kappa or 551 mu were grown at 27 °C, and that containing 51m1 kappa was grown at 30 °C. *Escherichia coli* B was grown in Beef Heart Infusion (Difco) on a rotary shaker at 37 °C.

Purification of endosymbionts. Class 7 kappas and 1038 kappa were purified from material released on lysis of host paramecia by elution from columns of ground filter paper (Whatman Ashless Tablets) (Preer & Preer, 1967). Other endosymbionts were purified using columns of epichlorhydrin triethanolamine cellulose (ECTEOLA; ICN Pharmaceuticals, Irvine, California, U.S.A.) as described by J. R. Preer, Jr, in a review by Sonneborn (1970). Class 51 kappas, 511 kappa and 51m1 kappa were collected in the ECTEOLA wash, but an additional wash with a high salt buffer (0.5 M-NaCl, 5 mM-Na₂HPO₄, pH 8.0) was needed to elute mu, nu and pi particles. Microscopic examination of purified endosymbiont preparations revealed less than 1 % contamination by other bacteria.

Preparation of DNA. Pellets of purified endosymbionts were suspended in saline EDTA (0.15 M-NaCl, 0.1 M-EDTA, pH 8.0) containing 0.5 mg lysozyme ml⁻¹ (purified sixfold; Miles Research Laboratories, Elkhart, Indiana, U.S.A.). An equal volume of saline EDTA containing 2 % (w/v) Sarkosyl (ICN Pharmaceuticals) and 50 µg Proteinase K ml⁻¹ (Beckman Instruments) was then added. Lysis was allowed to proceed at 60 °C for 30 min. DNA was purified and screened for the presence of circular DNA by ethidium bromide/CsCl buoyant density centrifugation (Dilts, 1976). Ethidium bromide was removed from the DNA by dialysis against Dowex Na⁺ resin (Thompson *et al.*, 1974) and CsCl was removed by exhaustive dialysis against 5 mM-NaCl.

Purified bacteriophage DNA from 562 kappa was kindly donated by Dr Judith A. Dilts (William Jewell College, Liberty, Missouri, U.S.A.).

Radioactive labelling of DNA, fragmentation and DNA-DNA hybridization were performed as previously described (Quackenbush, 1977*a*). Percentage hybridization was corrected for self-reaction of radioactively labelled DNA (tracer). Homology between heterologous DNA preparations was determined relative to the extent of hybridization between homologous tracer and driver (unlabelled DNA) DNA preparations.

RESULTS

The hybridization data (Table 2) show that the endosymbionts tested are genetically diverse and can be separated into at least four distinct groups.

Mu, nu and pi particles. Mu, nu or pi driver DNA preparations were at least 40 % homologous with tracer DNA from 540 mu or 1010 nu showing that mu, nu and pi are closely

Table 1. *Strains used*

Symbiont			Host species	Pre-lethal effect or killing activity	Origin of species
Binomial name	Greek letter	Stock			
<i>Caedobacter taeniospiralis</i>	Kappa (class 51)	47	<i>Paramecium tetraurelia</i>	Hump-killer	Berkeley, Calif., U.S.A.
<i>C. taeniospiralis</i>	Kappa (class 51)	51	<i>P. tetraurelia</i>	Hump-killer	Spencer, Ind., U.S.A.
<i>C. taeniospiralis</i>	Kappa (class 51)	169	<i>P. tetraurelia</i>	Hump-killer	Morioko City, Japan
<i>C. taeniospiralis</i>	Kappa (class 51)	Δ30	<i>P. tetraurelia</i>	Hump-killer	Australia
<i>C. taeniospiralis</i>	Kappa (class 7)	7	<i>P. biaurelia</i>	Spin-killer	Pinehurst, N. Carolina, U.S.A.
<i>C. taeniospiralis</i>	Kappa (class 7)	576	<i>P. biaurelia</i>	Spin-killer	Norwich, England
<i>C. taeniospiralis</i>	Kappa (class 7)	310	<i>P. biaurelia</i>	Vacuolizer	New Zealand
				Paralyzer	
<i>C. taeniospiralis</i>	Kappa (class 562)	511	<i>P. biaurelia</i>	Vacuolizer	Figate, Scotland
<i>C. taeniospiralis</i>	Kappa (class 562)	562	<i>P. biaurelia</i>	Vacuolizer	Milan, Italy
<i>C. taeniospiralis</i>	Kappa (class 1038)	1038	<i>P. biaurelia</i>	Spin-killer	Syktykar, U.S.S.R.
<i>C. taeniospiralis</i>	Kappa (class 51m1)	51m1	<i>P. tetraurelia</i>	Spin-killer	Stock 51
<i>C. taeniospiralis</i>	Pi	139	<i>P. tetraurelia</i>	None	Florida, U.S.A.
<i>C. conjugatus</i>	Mu	138	<i>P. octaurelia</i>	Mate-killer	Florida, U.S.A.
<i>C. conjugatus</i>	Mu	540	<i>P. primaurelia</i>	Mate-killer	Mexico
<i>C. conjugatus</i>	Mu	551	<i>P. primaurelia</i>	Mate-killer	San Francisco, Calif., U.S.A.
<i>C. falsus</i>	Nu	1010	<i>P. biaurelia</i>	None	Tennessee, U.S.A.

Table 2. *Percentage hybridization of ³H-labelled tracer DNA with unlabelled driver DNA*

Source of driver DNA	Source of ³ H-labelled tracer DNA					
	51 kappa	7 kappa	576 kappa	1010 nu	540 mu	562 kappaphage
47 kappa	60					14
51 kappa	100		4	1	6	
169 kappa	86					7
Δ30 kappa	41					5
7 kappa	<1	100	97	1	5	
310 kappa	<1	75	102			
576 kappa	<1	74	100	4	11	
511 kappa	15	38	41			76
1038 kappa	1	43	45			
51m1 kappa	<1	2	6			73
139 pi		3	6			
1010 nu	2			84	73	
138 mu				100	40	
540 mu				42	74	
540 mu	1		10	47	100	
551 mu				61	70	
562 kappaphage						100
51 kappa plasmid						13
<i>E. coli</i> B	<1	1	2	1	5	<1

related. Preparations of DNA from strains of mu, nu and pi were less than 12% homologous with any of the kappa DNA preparations tested.

Class 51 kappa particles. Driver DNA prepared from class 51 hump-killer kappas reassociated significantly only with tracer DNA from 51 kappa. Tracer DNA from 51 kappa did not reassociate with driver DNA prepared from non hump-killer strains of endosymbionts. Clearly hump-killer kappas are a genetically discrete group. They have no close affinities with other types of kappas, except that all are obligate endosymbionts of closely related species of *Paramecium* and can produce R bodies.

Kappa particles that infect P. biaurelia. Spin-killer (class 7) tracer DNA preparations from 576 kappa and 7 kappa were less than 10% homologous with driver DNA prepara-

Table 3. *Characteristics of Caedobacter and Pseudocaedobacter*

Species name	Type strain	Host organism	R body	Killing activity	Old species name
<i>Caedobacter taeniospiralis</i>	51 kappa	<i>P. tetraurelia</i>	+	Hump-killer	<i>C. taeniospiralis</i>
<i>Caedobacter varicaedens</i>	7 kappa	<i>P. biaurelia</i>	+	Spin-killer Paralyzer Vacuolizer	<i>C. taeniospiralis</i>
<i>Caedobacter pseudomutans</i>	51ml kappa	<i>P. tetraurelia</i>	+	Spin-killer	<i>C. taeniospiralis</i>
<i>Caedobacter paraconjugatus</i>	570 mu	<i>P. biaurelia</i>	+	Mate-killer	<i>C. conjugatus</i>
<i>Pseudocaedobacter conjugatus</i>	540 mu	<i>P. primaurelia</i> <i>P. octaurelia</i>	—	Mate-killer	<i>C. conjugatus</i>
<i>Pseudocaedobacter falsus</i>	1010 nu	<i>P. biaurelia</i> <i>P. tetraurelia</i> <i>P. pentaurelia</i>	—	None	<i>C. falsus</i> <i>C. taeniospiralis</i>
<i>Pseudocaedobacter minutus</i>	214 gamma	<i>P. octaurelia</i>	—	Vacuolizer	<i>C. minutus</i>

tions from 51 kappa, mu or pi endosymbionts. Kappas 576, 7 and 310 are apparently very closely related with a minimum homology of 74% between them. Driver DNA preparations from 1038 kappa and 511 kappa were 38 to 45% homologous with spin-killer (class 7) tracer DNA indicating that these organisms also share close genetic ties with class 7 kappa particles.

Class 51ml kappa. Driver DNA from 51ml kappa was not more than 6% homologous with any of the whole-cell tracer DNA preparations; this strongly suggests that 51ml kappa is not closely related to any of the other endosymbionts tested.

Extrachromosomal DNA from endosymbionts. Covalently closed, circular plasmid DNA was detected only in class 51 hump-killer kappas and in 139 pi. However, the plasmid from 139 pi was only about one-tenth the size of the plasmids from hump-killer kappa particles which ranged from 24×10^6 to 31×10^6 daltons (Quackenbush, 1977b).

Tracer DNA from kappaphage 562 (bacteriophage particles that occur in 562 kappa) showed only 13% homology with plasmid DNA from 51 kappa. When hybridized with whole-cell DNA preparations from other class 51 hump-killer kappas, 562 kappaphage tracer DNA showed less than 15% homology suggesting that there is only a distant relationship between these extrachromosomal elements.

DISCUSSION

The results presented here support the suggestion (Quackenbush, 1977a) that the genus *Caedobacter* should be revised. Brenner (1973) has suggested that the amount of deoxyribonucleotide sequence homology between strains of a given bacterial species should be at least 70%. Using this as a general guideline, the genus *Caedobacter* can be revised. However, the taxa in a good classification should be identifiable by practical laboratory procedures. With this in mind, it is proposed that *Caedobacter* be split into two genera on the basis of R body production, a trait that is easily observed by light microscopy. Organisms which can produce R bodies (commonly known as kappa particles) remain in the genus *Caedobacter*; those which cannot are placed in a new genus, *Pseudocaedobacter* (Table 3). The G+C content of the DNA of *Pseudocaedobacter* (35 to 39 mol %) is generally lower than that of *Caedobacter* (40 to 44 mol %).

The genus *Caedobacter* contains four species distinguished either by the types of killing activity they induce in sensitive strains of paramecia, or by the species of the *P. aurelia* complex that they infect (Table 1). The type species is *C. taeniospiralis*. Since 51 kappa is the type strain of *C. taeniospiralis* (J. R. Preer *et al.*, 1974), the class 51 hump-killer kappas retain this species designation.

Hybridization data reveal moderately close genetic relationships between class 7, class

562 and class 1038 kappas which are assigned to the new species *C. varicaedens*. Members of *C. varicaedens* can be identified by their killing activity and host (*P. biaurelia*). The type strain is 7 kappa.

Although 51ml kappa cannot be distinguished from members of *C. varicaedens*, either morphologically or by killing activity, the hybridization data indicate that it should not be included in *C. varicaedens*. Therefore 51ml kappa is placed in a new species, *C. pseudomutans*, which is characterized as a spin-killer that infects *P. tetraurelia*.

The only mate-killer known to produce R bodies, 570 mu, is the fourth species, *C. paraconjugatus*. Attempts to grow paramecia infected by 570 mu failed, so its genetic affinities with other strains of mu or kappa could not be determined.

The genus *Pseudocaeodobacter* is divided into three species according to killing activity. The type species is *Psc. conjugatus* comb.nov. It includes all strains of mate-killer endosymbionts except 570 mu, which is assigned to *C. paraconjugatus* because it can produce R bodies. The type strain of *Psc. conjugatus* is 540 mu.

Hybridization data show that pi and nu, which do not have any demonstrable killing activity, are closely related. Therefore they are placed in the same species, *Psc. falsus* comb.nov. The type strain of *Psc. falsus* is 1010 nu. In the original description of *Caedobacter falsus* (J. R. Preer *et al.*, 1974) the type strain was incorrectly given as 225 nu which does not exist.

Because they cannot produce R bodies, gamma are assigned to the species *Psc. minutus* comb.nov. This species can be distinguished from other species of *Pseudocaeodobacter* by its strong killing activity which is believed not to require cell contact between killer and sensitive paramecia. Although *Psc. minutus* is found only in low concentrations in host paramecia, it produces strong vacuolization in a broad range of sensitive paramecia from almost all species of the *P. aurelia* complex. The type strain of *Psc. minutus* is 214 gamma (Table 3).

The hybridization data presented here and the assignment of species to *Caedobacter* closely parallel the classification of kappa by Preer *et al.* (1972), which was based primarily on R body morphology, morphology of bacteriophage-like structures present within the kappa particles, and killing activity. Available evidence indicates that these characteristics are at least partially determined by extrachromosomal elements (Preer *et al.*, 1971; L. B. Preer *et al.*, 1974; Dilts, 1976, 1977). The apparent genetic diversity of *Caedobacter* (kappa) species and the probability that their major phenotypic traits are controlled by extrachromosomal elements could mean that *Caedobacter* consists of genetically diverse bacteria that carry genetically related extrachromosomal elements. This hypothesis is supported by the fact that tracer DNA from the bacteriophage-like particles occurring in 562 kappa (562 kappaphage) is 73% homologous with DNA from 51ml kappa (*C. pseudomutans*) which in turn is less than 15% homologous with any of the whole-cell tracer DNA used in this investigation. However, less than 14% of the kappaphage genome is homologous with plasmid DNA from class 51 hump-killer kappas (*C. taeniospiralis*). All hump-killer kappas carry extrachromosomal DNA in the form of covalently closed, circular plasmids with molecular weights ranging from 25×10^6 to 32×10^6 (Dilts, 1976, 1977; Quackenbush, 1977b) whereas the 562 kappaphage genome has a complexity (amount of non-repeated DNA) of about 40×10^6 (Quackenbush, 1977b). The extrachromosomal elements of kappa that carry genes essential for R body production thus vary in their physical structure, size and DNA sequence. The extrachromosomal elements of *Caedobacter* species may be as diverse genetically as are the species themselves. Possibly these extrachromosomal elements had separate origins but it is more likely that at least those portions coding for R body and toxin production had a common origin and have since diverged. These extrachromosomal elements could have evolved from one form (plasmid or bacteriophage) to another, or the genes for killing activity could have been transferred from their parent molecule (plasmid or bacteriophage DNA) to another species of DNA. A common origin for these genes is

suggested by the fact that R bodies and bacteriophage particles from *C. varicaedens* 562 (562 kappa), *C. varicaedens* 7 (7 kappa) and *C. taeniospiralis* 51 (51 kappa) are all immunologically cross-reactive (Singler-Bastiaans, 1975).

*Description of new and revised taxonomic groups within the
Caedobacteriaceae Preer et al. 1974*

Caedobacter Preer et al. 1974. Gram-negative, non-motile rods or coccobacilli 0.4 to 1.0 μm wide and 1.0 to 4.0 μm long. Up to 50% (usually less than 10%) of the cells in any given population contain single (rarely double) refractile inclusion bodies (R bodies). The R body is a proteinaceous ribbon, approximately 10 μm long, 0.5 μm wide and 13 nm thick, which is tightly rolled up within the cell (Anderson et al., 1964). Cells containing R bodies are toxic to certain strains of paramecia and are usually larger than cells that do not contain R bodies. All species are obligate cytoplasmic endosymbionts of *Paramecium biaurelia* or *P. tetraurelia*. Culture outside the host organism has not been achieved. The G+C content of the DNA ranges from 40 to 44 mol %. The type species is *C. taeniospiralis* Preer et al. 1974. These organisms are commonly known as kappa particles.

Caedobacter taeniospiralis Preer et al. 1974. Short, Gram-negative, non-motile rods or coccobacilli 0.4 to 0.7 μm wide and 1.0 to 2.5 μm long. Ingestion of R body-containing cells by sensitive paramecia usually results in the development of an aboral blister (hump) preceding the death of the paramecium. Obligate cytoplasmic endosymbionts of *P. tetraurelia*. The G+C content of the DNA is 41 mol %. The type strain is *C. taeniospiralis* 51 (found in *P. tetraurelia* stock 51).

Caedobacter varicaedens sp.nov. (L. adj. *varis* different; L. n. *caedes* act of killing; L. n. *varicaedens* different acts of killing). Gram-negative, non-motile, cigar-shaped rods 0.4 to 1.0 μm wide and 1.0 to 4.0 μm long. Different strains cause one of three pre-lethal effects in sensitive paramecia after ingestion. These are vacuolization, paralysis or reversal of spinning motion while swimming (spin-killing). Obligate cytoplasmic endosymbionts of *P. biaurelia*. The G+C content of the DNA is 40 to 41 mol %. The type strain is *C. varicaedens* 7 (found in *P. biaurelia* stock 7).

Caedobacter pseudomutans sp.nov. (Gr. adj. *pseudo* false; L. part. adj. *mutans* changing; M.L. n. *pseudomutans* false mutant). Gram-negative, non-motile, cigar-shaped rods approximately 0.5 μm wide and 1.5 μm long. Most notable pre-lethal effect on sensitive paramecia after ingestion is reversal of spinning motion while swimming. Obligate cytoplasmic endosymbionts of *P. tetraurelia*. The G+C content of the DNA is 44 mol %. The type and only strain is *C. pseudomutans* 51m1 (found in *P. tetraurelia* stock 51m1). Previously believed to be a mutant of *C. taeniospiralis* 51.

Caedobacter paraconjugatus sp.nov. (Gr. prep. *para* alike; L. part. *conjugatus* conjugated; M.L. n. *paraconjugatus* similar to mate-killers). Small Gram-negative, non-motile rods. Less than 1% of the cells contain R bodies, which are smaller than those found in the other species of *Caedobacter*. Ingestion of cells by sensitive strains of paramecia does not produce any observable toxic effects. Cell-to-cell contact between host and sensitive paramecia is required for toxic effects (mate-killing) to be observed in the sensitive paramecia. Obligate cytoplasmic endosymbionts of *P. biaurelia*. The type and only known strain is *C. paraconjugatus* 570 (found in *P. biaurelia* stock 570).

Pseudocaedobacter gen.nov. (Gr. adj. *pseudo* false; L. n. *caedes* act of killing; Gr. n. *bactrum* rod; M.L. n. *Pseudocaedobacter* false kappa particles). Gram-negative, non-motile rods 0.25 to 0.7 μm wide and 0.5 to 4.0 μm long. May or may not confer a killer trait upon their host paramecia. Do not produce R body-containing cells. Obligate cytoplasmic endosymbionts of *P. primaurelia*, *P. biaurelia*, *P. tetraurelia*, *P. pentaurelia* and *P. octaurelia*. The G+C content of the DNA ranges from 35 to 39 mol %. The type species is *Psc. conjugatus* originally described by Preer et al. (1974) as *Caedobacter conjugatus*.

Pseudocaedobacter conjugatus comb.nov. Previously *Caedobacter conjugatus* Preer *et al.* 1974.

Pseudocaedobacter minutus comb.nov. Previously *Caedobacter minutus* Preer *et al.* 1974.

Pseudocaedobacter falsus comb.nov. Previously *Caedobacter falsus* Preer *et al.* 1974. Obligate cytoplasmic endosymbionts of *P. biaurelia*, *P. tetraurelia*, and *P. pentaurelia*. The type strain is *Psc. falsus* 1010 (found in *P. biaurelia* stock 1010). Previously reported type strain (225) does not exist. Commonly known as nu and pi particles. *Psc. falsus* 51 and *Psc. falsus* 139 were previously believed to be mutants of *C. taeniospiralis*.

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