

# The killer trait of *Paramecium* and its causative agents<sup>1</sup>

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## Abstract

Some paramecia express the so-called killer trait: killer paramecia release toxic particles into the environment and these particles kill sensitive *Paramecium* cells after ingestion. These particles are bacteria belonging to the genus *Caedibacter*, endosymbionts of killer *Paramecium* strains. A peculiarity of *Caedibacter* is the presence of an unusual structure termed R-body (refractile body) due to its bright appearance in phase contrast microscopy. It is constituted of a proteinaceous ribbon which is typically coiled within the bacterial cell. In response to certain stimuli, e. g. a low pH, the R-body unrolls in a telescopic fashion and thereby produces a hollow tube, several times the length of the bacterium which is destroyed by the extension. R-bodies are prerequisite for the killer trait. Their structure and mode of action suggest that they act as a releasing system for an unidentified lethal toxin by delivering it from the bacterial cell to the cytoplasm of the sensitive *Paramecium* where it finally causes death. The expression of the killer trait confers a competitive advantage to *Caedibacter*-harbouring paramecia, but it also inflicts costs on both partners of this symbiotic association.

**Key words:** Competitive advantage, endosymbiont, horizontal gene transfer, intracellular bacteria, mutualism, parasitism, phage, R-body, symbiosis.

## Zusammenfassung

Bei einigen Paramecien ist das sogenannte Killer-Phänomen zu beobachten: Killer-Paramecien geben toxische Partikel in ihre Umgebung ab, welche nach Aufnahme sensitive *Paramecium* Zellen töten. Diese Partikel sind Bakterien der Gattung *Caedibacter*, Endosymbionten von Killer-*Paramecium* Stämmen. Eine Eigenart von *Caedibacter* ist das Vorkommen einer ungewöhnlichen Struktur, die wegen ihres hell leuchtenden Aussehens im Phasenkontrast R-Körper (refraktiler Körper) genannt wird. Dieser besteht aus einem Proteinband, welches innerhalb der Bakterienzelle auf typische Weise gerollt vorliegt. In Antwort auf bestimmte Stimuli, wie z. B. einen niedrigen pH Wert, entrollt sich der R-Körper teleskopartig und bildet so ein langes, hohles Rohr. Dieses hat eine Länge, die mehrere Male die des Bakteriums übertrifft, welches durch die Ausdehnung zerstört wird. R-Körper sind Voraussetzung für das Killer-Phänomen. Ihre Struktur und Wirkungsweise deuten an, dass sie als Freisetzungssystem für ein unidentifiziertes, tödliches Toxin agieren, dessen Freisetzung in das Cytoplasma des sensitiven *Parameciums* letztendlich zum Tode führt. Die Expression des Killer-Phänomens liefert einen Wettbewerbsvorteil für *Caedibacter*-infizierte Paramecien, es birgt aber auch Kosten für beide Symbiose-Partner.

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## 1. The killer trait of *Paramecium* and its causative agents

Many know *Paramecium* as a study object in biological courses at school and also in the beginners' courses at university. That is not without reason, as this unicellular organism offers an easy to handle model for the study of motility, behavior and the composition of eukaryotic cells.

*Paramecium* has long played a central role in our understanding of genetics as the original source of insight into cytoplasmic inheritance, RNA catalysis, telomere biology, gene scrambling, microRNAs, and DNA splicing (NANNEY 1980, GÖRTZ 1988, ASAI & FORNEY 1999, PREER 1997, NOWACKI et al. 2005, BEALE & PREER 2008). Finally, because they harbor a silent germline micronucleus and a transcriptionally active somatic nucleus in the same cell, ciliates – among them *Paramecium* – provide the closest possible unicellular analog to the biological architectures of multicellular species.

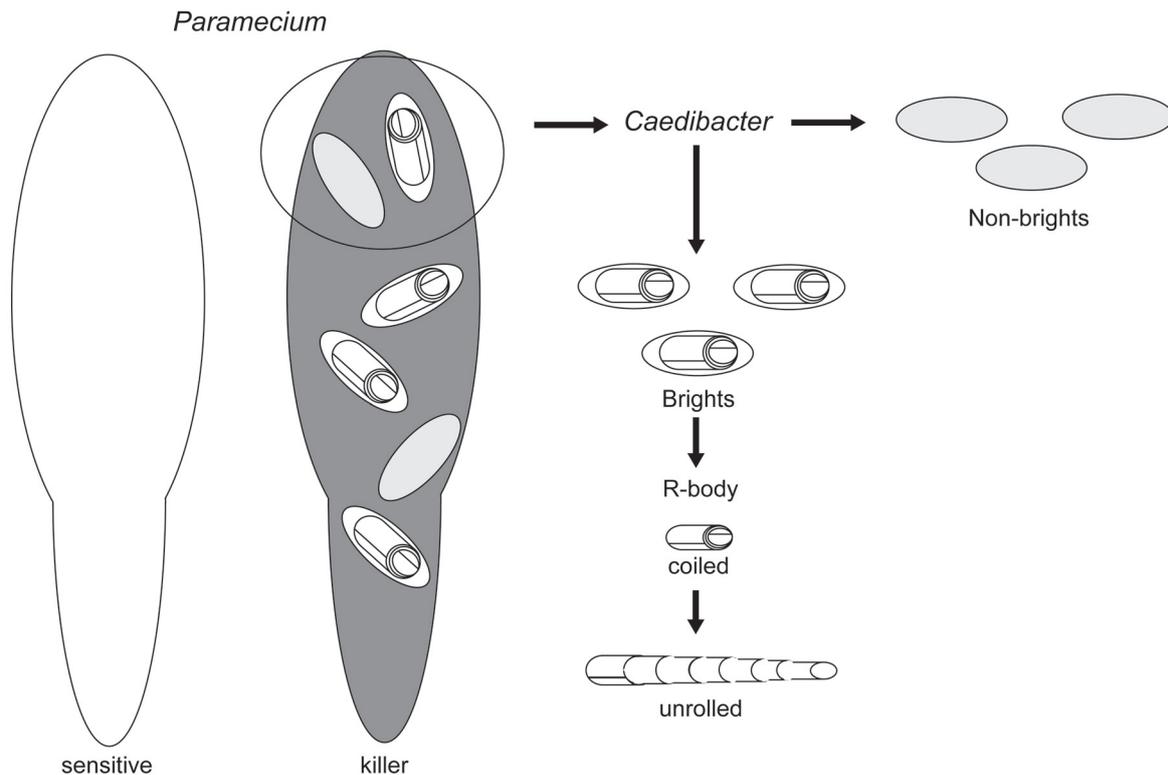
One field of *Paramecium* research is the characterization of the variety of symbiotic microorganisms it can harbour. Most natural populations of paramecia are indeed in-

fectured (GÖRTZ 2008). Protists can harbor a great variety of microorganisms, e. g. fungi, microsporidia, kinetoplastid flagellates, amoebas, and ciliates but most frequently bacteria (BALL 1969; GÖRTZ 1983, 1997; JEON 1991; HECKMANN & GÖRTZ 1991). Analyses of intracellular symbioses may reveal a wealth of unknown mechanisms to turn from previously free-living to an intracellular lifestyle.

Only few of the endosymbiotic bacteria of *Paramecium* have been characterized. Examples for well-known *Paramecium* endosymbionts are *Caedibacter* spp., which confer the killer trait to their host (see for reviews PREER et al. 1974b, POND et al. 1989, KUSCH & GÖRTZ 2006, SCHRALLHAMMER & SCHWEIKERT 2009).

### 1.1. Discovery of the killer trait of *Paramecium*

The phenomenon of the killer trait was discovered in 1938 by TRACY M. SONNEBORN (SONNEBORN 1938). He observed that *Paramecium* strains can be distinguished in “killer” and “sensitive” paramecia (Fig. 1): when sensitive individuals and members of a killer strain were mixed, the sensitive paramecia died within a few hours whereas



**Fig. 1.** Sensitive and killer paramecia differ only in the presence of endosymbiotic *Caedibacter*. Every *Caedibacter* population within a host cell presents two morphological different forms: reproductive non-brights and brights, which lost the capability to divide after the production of an R-body (refractile body). It is constituted of a coiled proteinaceous ribbon which can unroll in a telescopic fashion.

the killer cells survived. Therefore the killer trait in *Paramecium* (SONNEBORN 1938, 1943) was recognized as a competitive advantage for killer cells. AUSTIN (1948a, b) showed by transferring sensitive *Paramecium* cells into cell-free medium taken from a killer culture, that killing of sensitive paramecia results from exposure to toxic particles released from killers. Accordingly, killing needs no direct contact between killer and sensitive cells.

SONNEBORN'S studies of the killer trait gave rise to the description of one of the first examples for cytoplasmic inheritance. He discovered that the genetic determinants of the killer trait are located in the cytoplasm and referred to them as "plasmagenes" (SONNEBORN 1943). According to the taxonomic rules of his time, he termed these cytoplasmic factors "kappa particles".

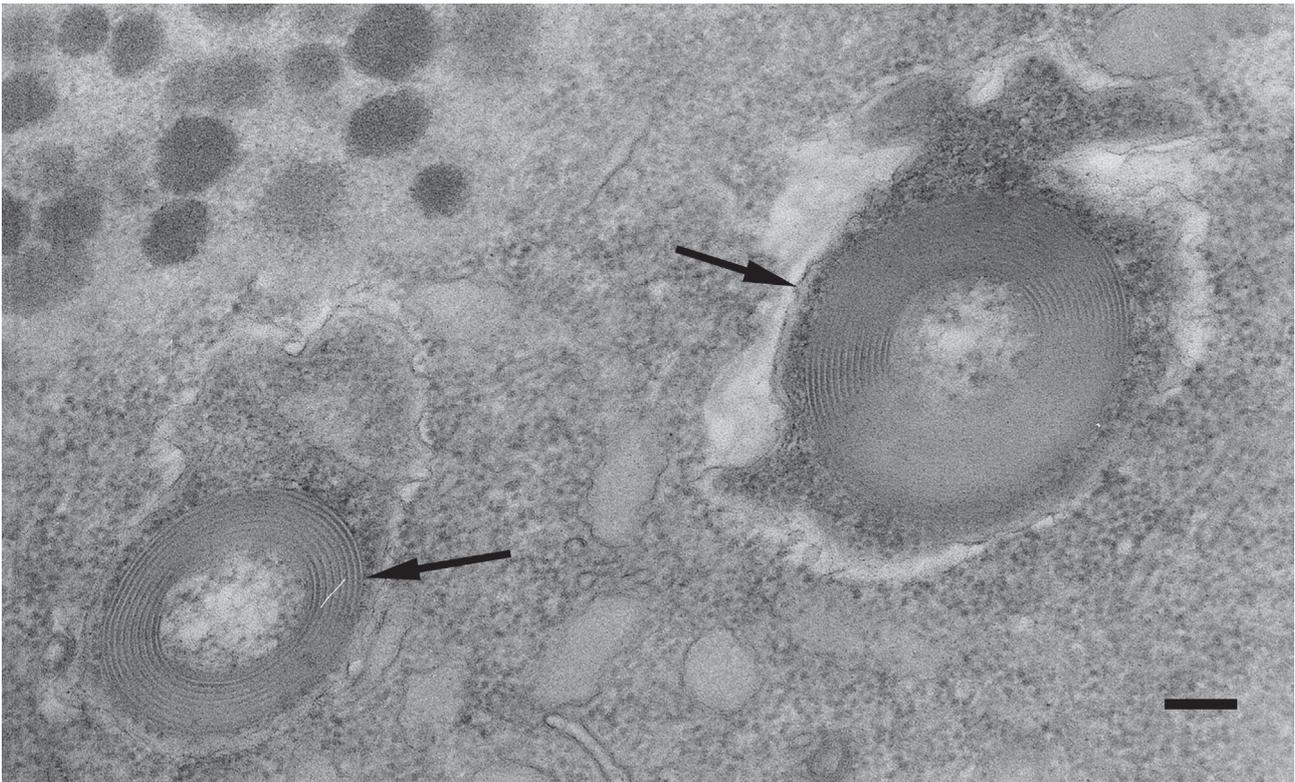
## 1.2. Nature of kappa particles

Later, kappa particles were identified as gram-negative bacteria (PREER 1950, HAMILTON & GETTNER 1958), and therefore had to be renamed (PREER & PREER 1982) using binominal nomenclature according to the Bacteriological Code. The so-called classes of kappa (PREER

et al. 1972) share several common features (see below), thus it was proposed to combine them in the therefore established new bacterial genus *Caedibacter* (detailed described by PREER & PREER 1982, GÖRTZ 2002, GÖRTZ & SCHMIDT 2005). All former kappa particles were arranged into four different species: *Caedibacter taeniospiralis* (PREER & PREER 1982), *Caedibacter varicaedens*, *Caedibacter pseudomutans*, and *Caedibacter paraconjugatus* (QUACKENBUSH 1982). A fifth species was included later with the description of *Caedibacter caryophilus* (SCHMIDT et al. 1987, EUZÉBY 1997). The common features leading to the establishment of the genus *Caedibacter* are an obligate endosymbiotic lifestyle, the expression of a killer trait in their host organism, and the production of R-bodies.

The R-body (refractile body, observed in phase contrast microscopy; Figs. 1–3) was first described by PREER & STARK (1953). It represents an unusual, highly complex structure in the cytoplasm of *Caedibacter* bacteria. Only a certain percentage of a *Caedibacter* population within a host produces R-bodies. These bacteria are termed brights due to their refractile inclusion whereas the other cells are referred to as non-brights (Fig. 1).

R-bodies were used as an easily recognizable diagnostic feature of the genus *Caedibacter* (Fig. 2). They are pro-



**Fig. 2.** Ultrathin section of two *Caedibacter taeniospiralis* cells in the cytoplasm of *Paramecium tetraurelia*, both harbor a coiled R-body in transverse section (arrows). – Bar: 100 nm.

teinaceous ribbons (Figs. 1, 2) rolled up to form a hollow cylinder of about 0.5  $\mu\text{m}$  in width and length (in the coiled state). Various criteria for species discrimination were provided by the R-body, like its dimensions, the shape of the ribbon tip (tapered or blunt), the potential association with phage-like particles and its unrolling mechanism (from the inside or the outside).

## 2. Ecological impact of the killer trait

The presence of *Caedibacter* endosymbionts turns *Paramecium* into a killer. *Caedibacter*-harboring paramecia have a selective advantage over aposymbiotic paramecia (LANDIS 1981), and it is natural selection that maintains the killer trait in *Paramecium* (LANDIS 1988). Distribution of *Caedibacter*-harbouring *Paramecium* strains is worldwide, infected stocks originate from North America, Central America, Europe, Japan and Australia (PREER et al. 1974b).

### 2.1. Impact on sensitive paramecia

Several members of the genus *Paramecium* are known to occupy overlapping ecological niches with respect to their prey organisms and climate preferences (GAUSE 1934; LANDIS 1981, 1988). Overlapping niches limit propagation and cause interspecific competition. Killer strains of *Paramecium* outcompete symbiont-free competitors of the same or closely related species.

Low numbers of *Caedibacter* cells are permanently released to the environment via the cytophyge of killer paramecia (NOBILI 1961, PREER et al. 1974b). Killing occurs if a single R-body carrying bacteria is ingested (AUSTIN 1948b) by *Caedibacter*-free and therefore sensitive *Paramecium*. Once ingested and incorporated into a food vacuole, the bright R-body is apparently important for toxin release (PREER et al. 1974b). Triggered by the acidification of the *Paramecium* phagosome, the R-body produces a long hollow tube, several times the length of the bacterium originally containing it. The unrolling results in extensive membrane breakage and disintegration, and vacuole contents are mixed with the cytoplasm of the sensitive *Paramecium* (JURAND et al. 1978). *Caedibacter* cell contents are also released.

Several experiments (PREER et al. 1953, SMITH 1961, MÜLLER 1962) suggest that not the mechanical unrolling of the R-body, like a stabbing, kills the sensitive *Paramecium*, but the action of a yet unidentified toxin produced by bright *Caedibacter*. The special shape and function of the R-body has led to the hypothesis that this structure functions as delivery system for the toxin (POND et al. 1989, SCHRALLHAMMER & SCHWEIKERT 2009).

Most likely, it is the action of this unidentified toxin which is lethal for the sensitive *Paramecium*. Why the R-body and the destruction of bacterial and vacuolar membranes are necessary for toxin activity is still unclear.

### 2.2. Costs for killer paramecia

Killer paramecia are bearing bacterial symbionts of the genus *Caedibacter* which produce R-bodies and most likely toxins. Whereas the killer cells themselves are resistant against the toxins, sensitive cells of the same or related *Paramecium* species are killed after ingestion of R-body producing *Caedibacter*. Therefore, symbiosis between *Paramecium* and *Caedibacter* is mutualistic under natural conditions, as far as the toxic symbiont confers its host selective advantages against competitors (LANDIS 1981, 1988; KUSCH et al. 2002). It is not a defensive strategy of the ciliate against predators (KUSCH et al. 2002, GÖRTZ et al. 2009). Under laboratory conditions however *Caedibacter* are described as energy parasites (KUSCH et al. 2002) taking up ATP directly from its host. Like *Rickettsia* and *Chlamydia*, they possess an ATP/ADP antiporter (LINKA et al. 2003) and therewith are capable of depriving their host of energy. The genes encoding for nucleoside-triphosphate-transporter were shown to be horizontally transferred among various obligate intracellular bacteria (LINKA et al. 2003).

Not all strains of a *Paramecium* species are capable to undergo symbiosis with *Caedibacter*. SONNEBORN (1943) described that the genetic condition of a potential host is crucial for the maintenance of *Caedibacter taeniospiralis* in a cell. It was shown that an allele K is needed for the bacteria to exist, while k in the homozygous state does not support the symbionts (SCHNELLER et al. 1959, BALSLEY 1967).

### 2.3. Effect of R-body production on *Caedibacter* cells

Population size of *Caedibacter* is regulated by the growth of the host. Under maximum growth conditions host cells can divide more often than their intracellular bacteria and these can become diluted after each cell division of the host organism. High numbers of *Caedibacter* can be obtained by lower division rates of the host, but overgrowth of the endosymbionts finally causes death of the host organism (SCHMIDT et al. 1987, KUSCH et al. 2002). Infections of new hosts are rare. Distribution of *Caedibacter* occurs with mitotic cell division or during conjugation of the ciliates (PREER et al. 1974b; LANDIS 1981, 1988).

*Caedibacter* populations are subdivided into two different types of cell morphology (Fig. 1), by phase-contrast microscopy recognizable as bright and non-bright cells.

R-body possessing cells are called brights because of its bright appearance inside the bacterium. *Caedibacter* without R-body are referred to as non-bright (PREER & STARK 1953). Bright cells arise from the non-bright forms (PREER et al. 1953, MÜLLER 1962). The change from non-bright to bright is apparently problematic: *Caedibacter* harbouring an R-body lose the capacity to reproduce. Only non-brights are reproductive and undergo cell division by normal binary fission.

In general, R-bodies are produced by just a fraction of the *Caedibacter* population. The proportion of bright forms typically varies from 1 to 35% but occasionally is as high as 50% depending on the particular *Caedibacter* strain, the nutritional status of the host, and presumably different exogenous factors. Starvation, exposure to ultraviolet light or treatment with certain antibiotics (e. g. mitomycin C) are some factors which lead to an increase of brights in *C. taeniospiralis* populations (PREER et al. 1974a, POND et al. 1989). These treatments, especially the last two, are also known as treatment for prophage induction.

### 3. The killer traits' causative agents

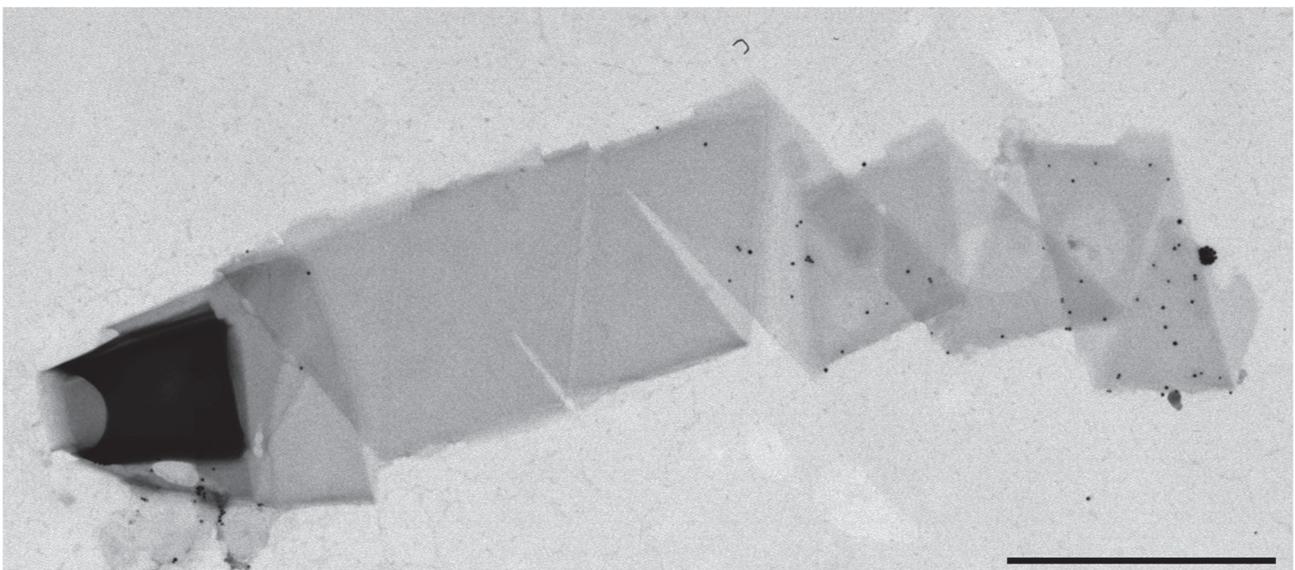
On the basis of the morphological characteristics mentioned above, five *Caedibacter* species were described so far. *C. taeniospiralis*, *C. varicaedens*, *C. pseudomutans*, and *C. paraconjugatus* are cytoplasmic endosymbionts in *Paramecium biaurelia* or *Paramecium tetraurelia* (sibling species in the *Paramecium aurelia* species complex). In contrast, the fifth species, *C. caryophilus*, was originally described as macronuclear symbiont of *Paramecium caudatum*.

It should be mentioned that, a side of *Caedibacter*, other killer symbionts occur in *Paramecium*; those do not express R-bodies (for review see PREER et al. 1974b).

#### 3.1. Endosymbionts of the genus *Caedibacter*

Several findings questioned if the characteristics used to discriminate the species of *Caedibacter* were well chosen. The ability to produce R-bodies can be lost spontaneously (FOKIN & GÖRTZ 1993), host specificity and cell localization of *Caedibacter* are not as preserved as assumed. KUSCH and co-workers (2000) demonstrated the presence of the same species, *C. caryophilus*, in different hosts (*Paramecium caudatum* and *Paramecium novaurelia*) and within different cell compartments (macronucleus, respectively cytoplasm). It was shown for *C. taeniospiralis* that R-bodies are plasmid encoded (QUACKENBUSH & BURBACH 1983, HERUTH et al. 1994, JEBLICK & KUSCH 2005).

First molecular biological indications that species of the genus *Caedibacter* are not closely phylogenetical related were reported with significant differences in GC-content and low DNA-DNA hybridization values among *Caedibacter* bacteria considered sister species (DILTS 1977; QUACKENBUSH 1977, 1978). Sequence characterization of the 16S rRNA gene and phylogenetic analyses of two species, *C. taeniospiralis* (BEIER et al. 2002) and *C. caryophilus* (SPRINGER et al. 1993, SCHRALLHAMMER et al. 2006), revealed that *Caedibacter* is a polyphyletic assemblage, with *C. taeniospiralis* belonging to the *Gammaproteobacteria*, closely related to the facultative intracellular pathogen *Francisella tularensis*, and *C. caryophilus*



**Fig. 3.** R-body of *Caedibacter taeniospiralis*, starting to unroll in a telescopic fashion. Negative contrast preparation. – Bar: 1  $\mu$ m.

belonging to the *Alphaproteobacteria*, clustering with the obligate *Paramecium* endosymbiont *Holospora*.

### 3.2. Components of the killer trait

The killer trait comprises three essential components, most likely provided by *Caedibacter* endosymbionts: the lethal toxin killing the sensitive *Paramecium*, the R-body (Fig. 3) which probably acts as a delivery device for the toxin, and the resistance mechanism protecting the *Caedibacter* host from the toxic action of its symbionts. Only if all three components are functional, the selective advantage of the killer trait and therewith of the *Caedibacter-Paramecium* symbiosis is warranted.

Toxicity in *Caedibacter* was found connected with the occurrence of R-bodies (DILTS & QUACKENBUSH 1986). Four different categories of killing have been described (SONNEBORN 1959, POND et al. 1989), namely hump killing, spin killing, vacuolization, and paralysis. The corresponding prelethal symptoms follow.

- Hump killing (*C. taeniospiralis*): a large aboral blister develops in affected cells.
- Spin killing (*C. pseudomutans*, *C. varicaedens* 7): affected cells swim with a direction of rotation opposite to the normal direction of rotation.
- Vacuolization (*C. varicaedens* 562): large vacuoles develop in affected cells.
- Paralysis (*C. caryophilus*): the affected cells cease swimming and only occasionally show weak avoidance reactions.

Most probably, these different symptoms are caused by different toxins produced by the different killer symbionts, as far as the R-bodies of the single species vary only in their morphology, not in their mode of action. Nature and mechanism of action of the toxin or the resistance towards it have not been identified yet (SCHRALLHAMMER & SCHWEIKERT 2009). As putative toxin of the hump killer *C. taeniospiralis*, a protein with homology to ATPases of the Soj/ParA family and membrane-associated ATPases involved in eukaryotic ATPase-dependent ion carriers was proposed (JEBLICK & KUSCH 2005). Immunity of *Caedibacter* hosts against killer trait related toxins is specific against a particular group of killers only. Thus, a hump killer strain is resistant to all other hump-killing strains, but it is susceptible to spin killers, vacuolizers, or paralyzers (POND et al. 1989).

### 3.3. R-bodies

The best studied component of the killer trait is the R-body. First observed by PREER & STARK (1953), the term R-body (ANDERSON et al. 1964, PREER et al. 1966) was chosen

because of its bright appearance in phase contrast microscopy. R-bodies are proteinaceous ribbons which are typically coiled within the bacterial cell (PREER & STARK 1953). The convoluted R-body (Fig. 2) represents a hollow cylinder which, in response to certain stimuli, can unroll in a telescopic fashion (Fig. 3). Although investigated intensively in the past, the detailed structure and function of the R-body is not yet completely understood.

R-bodies are very stable ribbons with different dimensions according to the producing bacterial species (POND et al. 1989, LALUCAT 1989). They are extremely resistant to denaturation and solubilization (PREER & PREER 1967, POND et al. 1989). Harsh treatments, generally known to denature proteins, do not affect the R-body ribbon. Their dimensions (reviewed by POND et al. 1989) range from < 10 µm to 20 µm (ribbon length) and 0.25 µm to 0.8 µm (ribbon width). The average ribbon thickness in the convoluted state is 11–16 nm (LALUCAT 1989). The total length of the R-body can sometimes differ, but ribbon morphology and ribbon width is conserved according to the bacterial species. In all *Caedibacter* species except *C. taeniospiralis*, phage-like particles were observed associated with the R-body ribbon (reviewed by POND et al. 1989).

Naturally, R-bodies unroll after the containing bacterium was incorporated into a sensitive *Paramecium* phagosome and the phagosome turned functionally into a lysosome. Bacterial cell lysis caused by acidification of the vacuole leads to a rapid change of pH. In vitro experiments with isolated R-bodies showed that unrolling can be triggered by a change in pH, but also by different physical and chemical conditions (PREER et al. 1966, BEDINGFIELD et al. 1984, GIBSON et al. 1987).

In *C. taeniospiralis*, the R-body's genetic determinants are localized on a plasmid (QUACKENBUSH 1983). Within this species, all strains contain a plasmid, pKAP (DILTS 1977). Because of variations in the number of mobile-element insertions, the size of pKAP varies between ca. 41.5 to 50.5 kb among different killer isolates. Interestingly, the plasmids of nonkiller *C. taeniospiralis* are much smaller (DILTS 1977). The region responsible for R-body production was determined by physical mapping (QUACKENBUSH & BURBACH 1983). The authors, actually investigating the toxin involved in the killer trait, cloned various fragments of the *C. taeniospiralis* plasmid pKAP47 in *Escherichia coli*. Instead of clones lethal for sensitive paramecia, they observed three clones with the ability to produce R-bodies, although with a rather low frequency (only 3% of the clonal population). Using deletions of the originally cloned fragment, it was possible to determine the R-body coding region (KANABROCKI et al. 1986). The R-body genes (*reb* genes) localized on the cloned pKAP47 fragment were sequenced (HERUTH et al. 1994) and later also the complete plasmid pKAP298 of *C. taeniospiralis*

298 (JEBLICK & KUSCH 2005). Of 63 identified ORFs (open reading frames), 23 have significant sequence homologies to known genes and of those 16 have phage-related functions. According to the authors, this high abundance indicates that pKAP298 might be derived from a former phage (already hypothesized by PREER et al. 1974b) which developed into an autonomous plasmid.

#### 4. Evolution of the killer trait

The *Paramecium-Caedibacter* symbiosis is a highly specialized association which results in the formation of the complex structure R-body. This structure is pre-condition of the sophisticated killer trait. The R-body, together with the toxin, increases the competitive advantage of *Caedibacter*-harbouring paramecia while eliminating *Caedibacter*-free *Paramecium* competitors (KUSCH & GÖRTZ 2006). An increased competitive advantage might support the maintenance of endosymbiotic *Caedibacter* and subsequently also the phages present in most *Caedibacter* species.

##### 4.1. Horizontal gene transfer of R-body encoding genes and the role of phages

The genus *Caedibacter* needs revision to account to new insights in the phylogenetic relationships of these endosymbionts obtained by molecular methods. Accordingly, these bacteria are only distantly related, but share a similar lifestyle and the ability to produce R-bodies. It seems very unlikely, that the genes encoding for R-body synthesis evolved independently in at least one member of *Gammaproteobacteria* and *Alphaproteobacteria*. Therefore, it can be assumed that *reb* genes have been horizontally transmitted.

Observations of phage particles in all *Caedibacter* species except *C. taeniospiralis* point to a possible involvement of phages as agents of the gene transfer event, especially regarding their close spatial association with the R-body ribbon. Considering the presence of a plasmid of possible phage origin in *C. taeniospiralis*, which encodes not only the R-body but also several phage related proteins, the hypothesis of bacteriophages as vectors for R-body productions seems even more likely. SONNEBORN (1959) even proposed that R-body synthesis is linked to prophage induction.

Additional support for a horizontal gene transfer of R-body encoding genes might be seen by the fact that also few free-living bacteria have been reported to produce these unusual structures. Again, no close phylogenetic relationship between members of *Caedibacter* and the free-living R-body producers *Hydrogenophaga taeniospira-*

*lis* (LALUCAT et al. 1982, WILLEMS et al. 1989), *Acidovorax avenae* subsp. *avenae* (WELLS & HORNE 1983, WILLEMS et al. 1992), *Rhodospirillum centenum* (FAVINGER et al. 1989), and *Marinomonas mediterranea* (HERNANDEZ-ROMERO et al. 2003) could be observed.

##### 4.2. Evolutionary advantage of the killer trait versus its costs

As more cases of chronic bacterial infections are characterized, the distinction between pathogenesis and mutualism has become increasingly blurred. Infection by a particular bacterium may be beneficial to a host under some circumstances, but harmful in other hosts or environments (DALE & MORAN 2006, KUSCH & GÖRTZ 2006). This is especially true for the *Paramecium-Caedibacter* symbiosis. The killer trait provides benefits to the killer paramecia by the elimination of competitors occupying the same or an overlapping ecological niche. But it includes also considerable costs implied by the maintenance of the energy parasite *Caedibacter*.

In *Caedibacter*, the killer trait enables the endosymbionts to occupy a comfortable niche within their *Paramecium* hosts and it ensures the maintenance of *Caedibacter* in a *Paramecium* population in two ways. First, the killer paramecia benefit from the presence of *Caedibacter* and second, the R-body producing bacteria kill potential hosts they cannot infect (e. g. hosts of the k allele phenotype). Of course this situation has its drawbacks, but regarding the fact that *Caedibacter* are barely infective (PREER et al. 1974b; LANDIS 1981, 1988; KUSCH et al. 2002), they apparently rely on vertical rather than on horizontal transmission.

Nevertheless, the killer trait is more costly for *Caedibacter* than for *Paramecium*. The endosymbionts “sacrifice” a part of their probably clonal population to produce R-bodies as far as the cells which built an R-body are not capable to divide any longer.

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