Comparative Study of Refractile (R) Bodies and Their Genetic Determinants: Relationship of Type 51 R Bodies to R Bodies Produced by *Pseudomonas taeniospiralis*

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The relationship of type 51 refractile (R) bodies to R bodies produced by *Pseudomonas taeniospiralis* was investigated. Proteins associated with type 51 R bodies were not serologically cross-reactive with proteins associated with R bodies from *P. taeniospiralis*. The genetic determinants for type 51 R bodies did not exhibit close homology with DNA sequences from *P. taeniospiralis*.

Refractile (R) bodies are inclusion bodies known to occur in only two genera of bacteria, *Caedibacter* and *Pseudomonas*. They are proteinaceous ribbons that, in their natural state, are rolled up, forming hollow cylinders resembling rolls of dialysis tubing. Until recently, all known species of R-body-producing bacteria were assigned to the genus *Caedibacter* (7-9). These bacteria, commonly known as kappa particles, are obligate endosymbionts of paramecia and confer killer traits upon their hosts. However, two free-living soil microorganisms, *Pseudomonas taeniospiralis* and *Pseudomonas avenae*, are now known to possess the ability to produce R-body-like structures (2, 4, 15). R bodies can be grouped into the following four basic types.

(i) Type 51 R bodies are produced by *Caedibacter taeniospiralis*. These bacteria confer the hump killer trait on their hosts (*Paramecium tetraurelia*). Synthesis of type 51 R bodies is required for expression of the hump killer trait by host paramecia (1a, 1b, 16). Inside the bacterial cell, these R bodies are up to 0.5 μm wide and 0.5 μm in diameter. Both termini of a type 51 R-body ribbon form acute angles. When unrolled, the R-body ribbon attains lengths of up to 15 μm (1). When exposed to a pH of less than 6.5, type 51 R bodies unroll from the inside in a telescoping fashion. This process can be reversed by returning the pH to neutrality. The genetic determinants of type 51 R bodies are plasmid borne and have been cloned and expressed in *Escherichia coli* (11).

(ii) Type 7 R bodies are produced by all other species of *Caedibacter*. These bacteria also confer killer traits (though not the hump killer trait) upon host paramecia. The size range of type 7 R bodies is similar to that of type 51. However, type 7 R bodies do not unroll in response to pH changes and have blunt or irregular outer termini (8). When type 7 R bodies do unroll, they do so from the outside like a rug. The genetic determinants of type 7 R bodies appear to be bacteriophages. Plasmids have not been detected in any strain of *Caedibacter* that produces type 7 R bodies (9; R. L. Quackenbush, unpublished observations).

(iii) R bodies produced by *P. taeniospiralis* are smaller than those produced by other R-body-producing bacteria. They are generally about 0.2 μm in length and 0.25 μm in diameter and resemble type 51 R bodies in that they unroll from the inside (3). However, unrolling is not pH dependent and is irreversible, as with type 7 R bodies. They further resemble type 7 R bodies in that their outer termini are blunt and their inner termini form acute angles (3).

(iv) R bodies produced by *P. avenae* are morphologically distinct from the other three types (15). The unrolled ribbon does not exhibit any parallel edges. The unrolled ribbon is widest at the midpoint and narrows progressively toward the termini where very acute (approximately 15°) angles are formed. These R bodies unroll in response to decreasing pH; however, in contrast to type 51 R bodies, they are reported to do so from the outside (6). In the rolled form, these R bodies are larger than the other types (0.5 to 0.8 μm long by 0.5 to 0.8 μm in diameter).

In this paper we present evidence that different R-body classes are not simply minor variations upon a basic type. DNA hybridization and immunological data presented here demonstrate that for *C. taeniospiralis* and *P. taeniospiralis*, there is considerable divergence in R-body proteins, as well as in the genetic determinants for these proteins.

DNA used in Southern blot hybridizations was purified from lysates of *E. coli* P678-54(pBQ65) and *P. taeniospiralis* by CsCl-ethidium bromide buoyant density centrifugation as previously described (11). Reaction conditions for restriction endonuclease digestions were according to the recommendations of the supplier (Bethesda Research Laboratories, Inc., Gaithersburg, Md.). Probe DNA (a 2.0-kilobase-pair *Hae*III-*Cla*I fragment of pBQ65 which codes for type 51 R-body synthesis) was prepared by nick translation with [α -³²P]deoxynucleoside triphosphates (ICN Pharmaceuticals Inc., Irvine, Calif.) as described by Rigby et al. (13). Transfer of DNA fragments from agarose gels to Nylon-66 membranes (GeneScreen, New England Nuclear Corp., Boston, Mass.) was performed as described by Southern (14). Hybridization of probe DNA to Southern blots was performed as described by Maniatis et al. (5).

Type 51 R bodies were purified from *E. coli* P678-54(pBQ65). Lysates of *E. coli* P678-54(pBQ65) were prepared as follows. Cell pellets obtained by centrifugation

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FIG. 1. Hybridization of pBQ65-derived type 51 R-body-coding probe with *P. taeniospiralis* DNA. (A) Ethidium bromide-stained gel containing restriction endonuclease digests of *P. taeniospiralis* DNA and pBQ65. (B) Autoradiograph of the Southern blot of the gel shown in panel A after hybridization with the 2,000-base-pair type 51 R-body-coding *ClaI-HaeIII* fragment from pBQ65. Lanes: a and f, *HindIII* digests of λ c1857 DNA (23, 9.6, 6.7, 2.3, and 2.0 kilobase pairs) and of pUC13 (2.7 base pairs), and *MspI* digest of pBQ65 (the only fragment visible [3.0 kilobase pairs], containing the complete type 51 R-body-coding sequence); b to e, restriction endonuclease digests (*HindIII*, *StuI*, *HincII*, and *PvuII*, respectively) of total cell DNA from *P. taeniospiralis*.

(4,420 $\times g$) of 250 ml of late-stationary-phase culture were suspended in 5 ml of 25% sucrose mix (50 mM Tris hydrochloride [pH 8.0]). Lysozyme was added to a final concentration of 2.5 mg/ml and incubated on ice for 15 min. DNase I and RNase were next added to a final concentration of 2 mg/ml each and incubated on ice for an additional 30 min. Then, 15 ml of 2% sodium lauroyl sarcosinate was added, with mixing followed by incubation on ice until significant

FIG. 2. Reaction of anti-type 51 R-body serum with proteins dissociated from purified R bodies. Proteins were boiled for 5 min in the presence of 2% sodium dodecyl sulfate–5% 2-mercaptoethanol, electrophoretically separated on a 7 to 20% sodium dodecyl sulfate gradient-polyacrylamide gel, and transferred to nitrocellulose. The blot was then exposed to anti-type 51 R-body serum. Bound antibody was visualized by a horseradish peroxidase-conjugated goat anti-rabbit antibody that catalyzes a colorimetric reaction. Sources of purified R bodies are indicated in the following lanes: A, *E. coli* P678-54(pBQ65); B, *P. taeniospiralis*; C, blank; D, *C. taeniospiralis* 51; E, *C. taeniospiralis* 116; F, *C. taeniospiralis* 169.

lysis was achieved. The lysate was pelleted by centrifugation (31,000 $\times g$) for 20 min. R bodies were purified from the lysate pellet by three rounds of centrifugation (1,500 $\times g$ for 45 min) through 10 to 55% sucrose gradients. After each round of sucrose gradient centrifugation, the R-body-containing fraction was collected with a Pasteur pipette, pelleted by centrifugation (31,000 $\times g$ for 15 min), and resuspended in 1 ml of 50 mM Tris hydrochloride (pH 8.0). R bodies were purified from *P. taeniospiralis* as previously described (3).

Rabbits were immunized by weekly intravenous injections of R bodies purified from sucrose gradients. Before injection, antigen was suspended in phosphate-buffered saline (0.8% NaCl, 0.115% Na_2HPO_4 , 0.02% KH_2PO_4 [pH 7.4]) to give a final concentration of 1.1 mg of purified R bodies per ml. Injections were delivered in 200- μ l doses. Proteins were electrophoretically transferred from polyacrylamide gels to nitrocellulose with a Trans-Blot apparatus (Bio-Rad Laboratories, Richmond, Calif.) according to the instructions of the manufacturer. After electrophoretic protein transfer, the nitrocellulose membrane was incubated in a blocking solution (3% gelatin, 0.05% Tween 20, 20 mM Tris hydrochloride, 0.5 M NaCl [pH 7.5]) for 30 min at room temperature and then rinsed in deionized water. The membrane was then incubated in primary antibody solution (1% antiserum, 1% gelatin, 0.05% Tween 20, 20 mM Tris hydrochloride, 0.5 M NaCl [pH 7.5]) at room temperature for 90 min, followed by a brief rinse in deionized water. After three 10-min washes in TTBS (20 mM Tris hydrochloride, 0.05% Tween 20, 5% NaCl [pH 7.5]), the membrane was incubated in the presence of the secondary antibody solution (goat anti-rabbit immunoglobulin G horseradish peroxidase conjugate [Bio-Rad], diluted 1:3,000 in TTBS containing 1% gelatin) for 90 min at room temperature, and rinsed in deionized water. Immune complexes were detected by the exposure of membranes to H_2O_2 and 4-chloro-1-naphthol to stain bound peroxidase.

The genetic determinants for type 51 R-body production by *C. taeniospiralis* have been cloned and expressed in *E. coli* (11). Restriction enzyme analysis (10) and DNA-DNA hybridization-Southern blot experiments (1a, 12; B. J. Cox,

FIG. 3. Reaction of anti-*P. taeniospiralis* R-body serum with proteins associated with R bodies. Procedures used were the same as described in the legend to Fig. 2, except that the Western blot shown above was exposed to anti-*P. taeniospiralis* R-body serum. Samples are indicated in the following lanes: a, R bodies purified from *P. taeniospiralis*; b, R bodies purified from *E. coli* P678-54(pBQ65); c, minicells from *E. coli* P678-54(pBQ65); d, molecular weight standards.

M.A. thesis, University of South Dakota, Vermillion, 1984) have demonstrated that type 51 R-body-coding sequences of the known strains of *C. taeniospiralis* are highly conserved. To determine whether the genetic determinants for *P. taeniospiralis* R bodies are closely related to those coding for type 51 R bodies, probe DNA from the recombinant plasmid pBQ65 (1b), which contains the coding sequence for type 51 R bodies, was hybridized with Southern blots containing restriction endonuclease digests of *P. taeniospiralis* DNA. The results (Fig. 1) of these hybridizations failed to reveal any homology between the type 51 R-body-coding probe and DNA from *P. taeniospiralis*, even when the stringency of the hybridization reaction was lowered to 50%.

Reactions of anti-R-body sera with Western blots containing homologous antigen (pBQ65-derived type 51 R bodies [Fig. 2] or *P. taeniospiralis* R bodies [Fig. 3]) revealed exactly the same polypeptide patterns as observed in Coomassie blue-stained polyacrylamide gels containing samples from the same sources (data not shown). The extreme difference between the polypeptide profiles generated by gel electrophoresis of purified type 51 R bodies and of *P. taeniospiralis* R bodies further indicated that there are significant differences in the structure and probably the manner in which these R bodies are assembled.

To determine whether there are any close structural similarities shared by proteins associated with type 51 R bodies and proteins associated with R bodies from *P. taeniospiralis*, we initiated a reaction of antisera against these R bodies with Western blots containing R-body-associated proteins. Antisera raised against type 51 R bodies coded for by pBQ65 reacted strongly with proteins associated with purified R bodies from *E. coli* P678-54(pBQ65) and three strains of *C. taeniospiralis* (Fig. 2). The protein profiles visualized by these four reactions were indistinguishable. This result further demonstrated that type 51 R bodies produced in *E. coli* are not perceptibly different from those produced in *C. taeniospiralis*. No reaction of the anti-type 51 R-body serum with proteins associated with R bodies purified from *P. taeniospiralis* could be detected (Fig. 2). Antiserum raised against purified R bodies from *P. taeniospiralis* reacted strongly with proteins associated with those R bodies (Fig. 3). However, only a limited reaction was observed between anti-*P. taeniospiralis* R-body serum and proteins associated with type 51 R bodies that were purified from *E. coli* P678-54(pBQ65) (Fig. 3). It is not clear whether this limited reaction was due to similarities between the two R-body types or to nonspecific binding perhaps caused by the presence of a cross-reactive contaminant.

These results demonstrate that type 51 R bodies are not closely related to those produced by *P. taeniospiralis*. This conclusion applies not only to the proteins associated with these R bodies but to their genetic determinants as well. These results are supported by the observation that R bodies from *C. taeniospiralis* 51 are not immunologically cross-reactive with R bodies purified from *P. taeniospiralis* as determined by immunogold labeling and electron microscopy (B. Wells and I. Gibson, personal communication). Wells and Gibson further observed that type 51 R bodies are not immunologically cross-reactive with R bodies purified from *P. avenae*. Wells et al. (B. Wells, J. Lalucat, and I. Gibson, *Micron Microsc. Acta*, in press), also using immunogold labeling and electron microscopy, reported that there may be weak cross-reactivity of anti-*P. taeniospiralis* R-body serum with R bodies purified from *P. avenae*. Preliminary observations indicate that (i) with the single exception of *Caedibacter varicaedens* 1038, the genetic

determinants of type 51 R bodies are not closely related to DNA sequences from *Caedibacter* strains that produce type 7 R bodies (Cox, M.A. thesis); and (ii) proteins associated with type 51 R bodies exhibit no convincing immunological cross-reactivity with proteins associated with type 7 R bodies (J. A. Kanabrocki, Ph.D. dissertation, University of South Dakota, Vermillion, 1985). It is, therefore, our conclusion that the four variations on the basic R body type presented at the beginning of this paper truly reflect four distinct types of structures. Since type 51 and type 7 R bodies are both known to occur only in obligate endosymbionts of paramecia, it is highly likely that these structures share a common evolutionary origin. If the pseudomonad R bodies also share this common origin (which we believe is most probable), the question arises whether the organisms discussed here share a common evolutionary origin or whether the ancestral determinants for R-body production have passed among unrelated bacteria by one or more types of genetic transfer.

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