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MINIREVIEW

Genetic Manipulation of Rickettsiae: a Preview

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The genus *Rickettsia* includes the causative agents of some of the most historically significant and severe bacterial diseases of humans. These include *Rickettsia prowazekii*, the agent responsible for epidemic typhus, and *R. rickettsii*, the agent of Rocky Mountain spotted fever. Additional species, including *R. typhi*, responsible for murine or endemic typhus, and others, responsible for a variety of spotted fevers occurring worldwide, have also been identified (8, 12). Increasingly, as new rickettsioses are identified and the disease potential of well-known rickettsial diseases are assessed, the rickettsiae are being recognized as emerging or reemerging pathogens (18). For example, the most notorious rickettsial disease, epidemic typhus, considered by many to be a disease of historical importance only, remains capable of producing severe morbidity and mortality in those populations where the classical precipitating factors of war, poverty, and poor vector control exist. Recent outbreaks testify to the continued threat that *R. prowazekii* poses (17, 20). Even the successes resulting from public hygiene in the United States have failed to eliminate *R. prowazekii*. In addition to individuals that carry rickettsiae for many years after the initial infection and then experience a recrudescence of the disease (Brill-Zinsser disease), a zoonotic reservoir, the flying squirrel, that maintains *R. prowazekii* has been identified in the eastern United States (5). In addition, all of the rickettsial diseases pose a diagnostic challenge for the physician, with failure to properly diagnose and treat these diseases resulting in high mortality.

As described in a number of excellent reviews and books dealing with the biology of rickettsiae (8, 13, 22, 23, 25, 26, 28), rickettsiae are gram-negative, obligate, intracellular parasitic bacteria that are able to grow only within the cytoplasm or, occasionally, the nucleus of a variety of eukaryotic host cells. Thus, they differ from the obligate intracellular bacteria of the genera *Chlamydia*, *Coxiella*, and *Ehrlichia* that grow within phagosomes or phagolysosomes. Another distinguishing characteristic of these novel bacteria is their association with arthropod vectors (4). Lice, fleas, ticks, and mites serve as vectors for one or more rickettsial species.

Rickettsiae enter host cells by induced phagocytosis, a process that requires active participation by both the rickettsia and the host cell and the appearance of a phospholipase A activity (24). The rickettsiae rapidly escape from the phagosome and enter the host cell cytoplasm, where they are able to exploit high-energy compounds present there, such as ATP, using specialized transport systems (27, 29). However, they are not strict energy parasites and retain the ability to generate ATP via an intact tricarboxylic acid cycle and oxidative phosphorylation. Some members of the genus, most notably *R. rickettsii*, are capable of polymerizing actin for movement within and between cells while others, such as *R. prowazekii*, do not exhibit this property (7, 9, 10). The end result of rickettsial growth, and the basis of their pathogenicity, is lysis of the host cell. Delineating the mechanisms involved in this unique, obligate, intracytoplasmic parasitism is the goal of current studies.

GENETIC ANALYSIS

The rickettsiae offer a fascinating model of intracellular parasitism. Despite the problems associated with studying an obligate intracellular parasite, the rickettsiae are amenable to sophisticated studies, as exemplified by the brief description above. Great strides have been made in our understanding of the invasion and lysis processes, the biochemistry of novel transport systems, and, with gene cloning and sequencing, our first look at rickettsial gene expression. Of great importance was the publication of the *R. prowazekii* genome sequence (1). This work revealed that *R. prowazekii* codes for 834 protein-coding genes, many of which could be annotated based on protein homologies, and the presence of a high percentage of noncoding DNA, hypothesized to be the result of ongoing reductive evolution. Phylogenetic analysis revealed that *R. prowazekii* is closest to mitochondria of all the currently sequenced bacterial genomes, making it a prime evolutionary model as well as an important pathogen. Thus, the *R. prowazekii* genome map and the approaching completion of additional rickettsial genome sequences (e.g., *R. conorii*, Genoscope [http://www.genoscope.cns.fr]) provide a smorgasbord of features and gene targets of great interest that await examination. Unfortunately, progress in correlating rickettsial genes and gene function has been hampered by the lack of genetic tools.

Examination of the rickettsiae at the morphological and genome levels reveals no major barriers to the development of rickettsial genetic systems. For example, the rickettsial cell wall displays no unusual properties. The rickettsiae exhibit a typical gram-negative morphology and composition and do not appear to generate different morphological forms. Thus, methods for introducing DNA into gram-negative bacteria, such as electroporation, should be effective for the rickettsiae. Indeed, all three mechanisms of bacterial genetic exchange, namely, conjugation, transduction, and transformation, can be considered. However, the clonal nature of rickettsial growth within host cells and the apparent absence of rickettsial plasmids indicate that natural conjugal mechanisms are absent. Similarly, rickettsial bacteriophages have not been identified. While this does

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not preclude the application of characterized bacterial conjugation and transduction systems to rickettsial species, at the present time, transformation provides the most feasible and direct approach for introducing DNA into rickettsiae. Fortunately, the rickettsiae can be isolated from the host cells and remain viable extracelluarly for hours, thus allowing time for manipulations. Following entrance of DNA into their rickettsiae, restriction enzyme destruction of the transforming DNA could certainly present a problem, although genes coding for enzymes homologous to well-characterized restriction systems have not been identified in the genome sequence. Early feasibility studies revealed that plasmid DNA isolated from *Escherichia coli* and electroporated into rickettsiae was lost at a rapid rate but could still be detected at low levels several days following electroporation. Finally, a number of years ago it was established that *R. prowazekii* possesses a *recA* gene coding for a product that can complement recombinational deficiencies in *E. coli* mutants (6), and additional genes involved in homologous recombination have subsequently been identified in the genome sequence. Thus, in the critical areas of DNA uptake and homologous recombination, the rickettsiae do not appear to present insurmountable barriers to the development of genetic systems.

Why has it taken so long for rickettsial genetic systems to be developed? The answer to this question is simple: the rickettsiae are obligate, intracellular parasites, a life style that places restrictions on any genetic system. For example, electroporation of rickettsiae requires lysis of infected host cells and isolation and preparation of competent rickettsiae. Following electroporation, the rickettsiae are allowed to reinfect viable host cells. Since a transformed rickettsia unable to enter a host cell is sentenced to death, manipulation of host-free rickettsiae must not prevent the subsequent infection of host cells. Also, the identification and isolation of rickettsial transformants have been difficult due to a lack of selectable markers. Antibiotics that are effective against the intracellular rickettsiae, such as tetracycline and chloramphenicol, cannot be used in genetic studies because of their importance in the clinical treatment of rickettsial diseases. Finally, the rickettsiae are class III pathogens that require special facilities and protocols for their study. This has restricted the number of investigators interested in addressing these problems and examining the exciting aspects of rickettsial intracellular growth.

Recently, the feasibility of the transformation approach was established for two rickettsial species. As might be expected from the discussion above, the successful protocols were relatively straightforward. Host cell-free rickettsiae were isolated, suspended in sucrose or glycerol, and electroporated at field strengths of approximately 17 to 24 kV/cm. The field strengths used for rickettsiae are higher due to the size of the rickettsiae (10% the volume of *E. coli*). Following electroporation, the rickettsiae were then allowed to infect host cells and their growth was monitored. Rachek et al. (16) demonstrated that DNA could be introduced into a rickettsial cell via electroporation and could subsequently recombine into the genome by homologous recombination. A single, basepair mutation that differed between the *rpoB* genes of rifampin-sensitive and rifampin-resistant strains of *R. prowazekii* was identified and used as a selectable marker for the transformation experiments. Silent mutations were introduced to distinguish transformants and spontaneous mutants. Troyer et al. (21) demonstrated transformation of *R. typhi* using expression of the green fluorescent protein (GFP) to detect recombinants. For the *R. typhi* experiments, transformation was accomplished with a PCR product in which the GFP gene was translationally fused to the *rpoB* gene. Expression of GFP in a rickettsial population was determined by flow cytometric analysis. Curiously, at least 10% of the unselected population expressed GFP 8 days after electroporation. In contrast, in the *R. prowazekii* transformations with rifampin selection, rickettsial numbers were reduced to a level below the level of visual detection at 7 days before the appearance of a rifampin-resistant population at 11 days, indicating a much lower transformation frequency. For the *R. prowazekii* system, transformants could be detected only after selection for the resistance phenotype. Whether this difference is species related or related to the specific *rpoB*-GFP fusion construct used in the *R. typhi* experiments is unknown. A minimal transformation frequency for *R. prowazekii* can be estimated from the experiments in which transformants were isolated and homologous recombination of the transforming DNA into the rickettsial genome was confirmed. The frequency would be 1×10^{-8} since at least one transformant was obtained from populations of approximately 1×10^{8} rickettsiae at the time of selection (estimated by counting the number of rickettsiae in 100 host cells following electroporation and reinfection). Determining a transformation frequency would be a monumental task considering the difficulties in enumerating rickettsiae, the indicated very low frequency seen in the selection experiments, the inability to use large numbers of rickettsiae per host cell during initial infection and selection, the imperfections of selection, and the necessity of distinguishing spontaneous mutants and transformants when the two rates are similar.

SELECTION

In the case of *R. prowazekii* transformation, the selection of transformants with rifampin was effective due to the sensitivity of *R. prowazekii* to this antimicrobial agent and the fact that the experiments were gene replacements, since sensitivity is usually dominant when both a rifampin-resistant gene and a rifampin-sensitive gene are present within a cell (14). Thus, while rifampin selection was an excellent model for characterizing rickettsial transformation conditions, the usefulness of a rifampin-resistant allele of *rpoB* as a selectable gene for more-extensive genetic experiments is limited. This prompted a search for alternate selections that would be more versatile, namely, an antibiotic to which *R. prowazekii* is sensitive and a resistance mechanism that preferably would operate by inactivating the antibiotic. Recently, erythromycin resistance was used successfully as a selectable marker in an *R. prowazekii* transformation (15). *R. prowazekii* is sensitive to erythromycin in in vitro assays with MIC determinations varying over a range of 0.125 to 2 μg/ml (11, 19). The *R. prowazekii* Madrid E isolate that was used in the original transformation experiments described above is sensitive to erythromycin with an MIC of 0.2 μg/ml when grown in mouse L929 fibroblast cells. In addition, a gene (*ereB*) coding for a product with an esterase activity that inactivates erythromycin has been identified in *E. coli* (2, 3). Insertion of the *ereB* gene into the rickettsial chromosome and isolation of a cloned rickettsial transformant have been achieved, establishing the feasibility of using this selection to generate knockout mutants of *R. prowazekii* (15).

Two basic methods for generating knockout mutants in rickettsiae have now been established. One relies on a single crossover event (insertion of the *ereB* plasmid into a selected region of the genome) while the other involves a double-crossover event with DNA replacement (substitution of a rifampin-sensitive allele for a resistant allele or substitution with an *rpoB*-GFP gene fusion). These classic bacterial methods for generating knockouts provide a straightforward result when knockout mutants are obtained. However, the rickettsial sys-

tem, unlike those of free-living bacteria, is more difficult to interpret if negative results are obtained. Due to the enormous effort and substantial time it takes to perform these experiments with rickettsiae, the investigator needs to feel confident of negative results (i.e., the inability to generate a knockout of a specific gene) and the assignment of a gene into the essential category. For most bacterial genetic systems, this is not a worry. In systems where one can perform a knockout experiment overnight, it is possible to perform multiple experiments in a very short time and feel confident that a negative experiment is truly negative. In such a rapid system, multiple, independent experiments could be performed before a single rickettsial transformation yielded transformants. Thus, for rickettsial systems, it will be essential to determine that the inability to obtain knockouts is not due to an inability to recombine at a specific site. This will require parallel experiments to demonstrate the insertion of DNA at the site that will not disrupt the gene.

THE FUTURE

The rickettsiae have sometimes been dismissed by investigators as degenerate bacteria deficient in important bacterial activities (i.e., those of *E. coli* and *Salmonella*) rather than being recognized as highly evolved organisms exquisitely adapted to the intracytoplasmic environment. Probably, such attitudes result from the difficulties associated with growing these biological-level-3 organisms and with the absence of genetic tools for correlating genes and gene function. With the recent publication of the *R. prowazekii* genome sequence (1) coupled with the successful transformation of *R. prowazekii* and *R. typhi*, such reticence should be eliminated and the feasibility of other genetic techniques should be assessed. For example, can transposon systems be used to generate random rickettsial knockouts?

The rickettsiae offer an intriguing model of intracellular parasitism. They are capable of infecting a wide range of eukaryotic cells and hosts as different as arthropods and humans. They can exploit the intracellular environment by expressing specialized transport proteins and can survive in some cases for years in healthy human hosts. Evolutionarily, they are currently recognized as the closest living bacterial relative of mitochondria. Analysis of these organisms at the genetic level is sure to provide valuable information on rickettsial pathogenicity, obligate intracellular parasitism, and bacterial evolution.

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