Transformation of *Rochalimaea quintana*, a Member of the Family *Rickettsiaceae*

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Rochalimaea quintana is the only member of the family *Rickettsiaceae* that can be grown in vitro. Because of its relationship to the other members of this family, techniques developed to transform *R. quintana* might be applicable to the obligate intracellular bacteria of the *Rickettsiaceae*. These procedures are critical to understanding mechanisms of pathogenesis and the nature of obligate intracellular growth. A transformation procedure for *R. quintana* has been established by using electroporation techniques. Several cosmids or plasmids with replicons RK2 and RSF1010 have been successfully used to transform this organism. Transformants were obtained by selection for antibiotic resistance to chloramphenicol or kanamycin. Plasmid retention and replication has been verified by Southern blot analysis and chloramphenicol acetyltransferase assay. Experimentation with different voltage field strengths and pulse times indicate that 12.5 kV/cm at 10 ms (25 μ F and 400 Ω) was optimal, giving a transformation frequency of $\sim 0.3\%$ and 3×10^5 transformants per μ g of DNA.

The family *Rickettsiaceae* contains a number of important human pathogens that are associated with arthropods. The members of this family differ markedly and have been separated into three genera: *Rickettsia*, *Coxiella*, and *Rochalimaea* (29).

Both *Rochalimaea quintana* and *Rochalimaea vinsonii* can be cultivated axenically and appear to grow in epicellular locations within their eucaryotic hosts (8, 27). Members of this genus appear to be more closely related to some species of the genus *Rickettsia* than to *Coxiella burnetii*. Indeed, *C. burnetii* does not appear to be related to either *Rickettsia* or *Rochalimaea* species (24, 25). *R. quintana* has been shown to have metabolic and nutritional properties similar to *Rickettsia* spp. (27). In support of these findings, studies of the genetic relatedness of *Rochalimaea* and *Rickettsia* species by DNA-DNA hybridization analyses reveal a small but significant degree of hybridization (16). Recently, genetic analyses utilizing highly conserved rRNA sequences have confirmed the relationship between these two genera (26). Classical genetic studies of the genera *Rickettsia* and *Coxiella* have been severely restricted because of difficulties in manipulating these obligate intracellular organisms. The ability to transform these organisms would allow the introduction of specific mutations or genes and transformants could be examined for altered biological properties in vivo. It was reasoned that if procedures could be developed to successfully transform *Rochalimaea quintana*, similar conditions might transform the more intractable members of the genus *Rickettsia*.

Because of the slow growth rates of *Rochalimaea* spp. and difficulties involved in selecting for transformants, a technique giving the highest efficiency of transformation is needed. Electroporation (2, 5, 14) appears to provide the best method for transforming members of the *Rickettsiaceae*. This method has been used to introduce DNA into bacterial cells at efficiencies approaching 5×10^9 transformants per μ g of plasmid DNA (2). Although *C. burnetii*, a

member of the *Rickettsiaceae*, contains plasmids that could be used to carry specific genes between organisms (19–21), no plasmids have been reported in the other members of the *Rickettsiaceae*. We therefore attempted to transform *R. quintana* using electroporation to introduce several plasmids with different origins of replication. We have demonstrated, by using plasmid-encoded antibiotic genes, that plasmid DNA replicates and is expressed in *R. quintana*.

MATERIALS AND METHODS

Bacteria. *Rochalimaea quintana* (trench fever) ATCC VR-358 Fuller strain was obtained from the American Type Culture Collection, Rockville, Md. Cells were grown on enriched chocolate agar plates (28), a stock was prepared, and the cells were frozen at -70°C . A new working culture was prepared monthly from the stock culture to minimize mutations and/or contamination. *Escherichia coli* DH5 and HB101 (Bethesda Research Laboratories, Inc., Bethesda, Md.) were used to prepare cosmid and plasmid stocks (13).

Growth and preparation of *Rochalimaea quintana*. Solid medium containing agar was prepared as previously described (28). When required, stock kanamycin (KAN) (25 mg/ml) or chloramphenicol (CAM) (34 mg/ml) was added aseptically to a final concentration of 10, 15, or 20 μ g/ml or 1 and 2 μ g/ml, respectively. Conditions for growth were 35°C , 95% air and 5% CO_2 , and 95 to 100% relative humidity.

After 4 to 5 days of growth, rickettsia were aseptically harvested from plates into rickettsia suspension medium (0.22 M sucrose, 0.05 M KH_2PO_4 , 0.005 M MgSO_4 , 0.002 M succinate, 0.002 M glutamate; pH adjusted to 7.2). The cells were suspended at room temperature into a 40-ml tube, and centrifuged in a JA-20 rotor (Beckman Instruments, Inc., Palo Alto, Calif.) at 6,000 rpm for 10 min at room temperature. The pellet was suspended and incubated for 20 min in trypsin (0.25% trypsin in HEPES-sucrose buffer [8 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, 272 mM sucrose; pH 7.4]) at 37°C to remove residual electrolytes. Treated cells were centrifuged at 4°C and washed twice in chilled HEPES-sucrose buffer. The cell concentration was adjusted, and 1×10^{10} to 5×10^{10} cells were

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suspended in 1 ml of chilled 10% glycerol and left on ice until they were electroporated.

A 40- μ l suspension of *R. quintana* was loaded into a 0.2-mm-wide electroporation cuvette and chilled on ice, and the desired quantity of DNA was added. After allowing the cells to equilibrate for 5 to 10 min (14), electroporation was performed by using a Gene Pulser unit (Bio-Rad Laboratories, Richmond, Calif.) set at voltage field strengths from 6 to 12.5 kV/cm and at pulse times of 5, 10, and 25 ms; these pulse times correspond to 200, 400, and 1,000 Ω at a capacitance of 25 μ F, respectively. The cells were immediately removed from the cuvette by rinsing with 1 ml of sterile broth medium (28) and incubated at 35°C in 95% air and 5% CO₂ without shaking. After 5 h, the cells were plated onto selective media. Transformed colonies were detected after 12 to 17 days.

Vector preparation and detection. Plasmids and cosmids were prepared by standard methods (4, 13). To obtain DNA from *R. quintana*, rickettsia were aseptically harvested from plates into rickettsia suspension medium after 4 to 5 days of growth. The cells were harvested at room temperature and pelleted by centrifugation. Chromosomal DNA from *R. quintana* was prepared by lysing cells with thermolysin and sodium dodecyl sulfate, centrifuging the cell lysate in a CsCl gradient at 55,000 rpm in a Vti 80 vertical rotor (Beckman) for 7 h, and then extracting and precipitating the DNA (19).

For Southern blot (23) analysis, vector DNA from *R. quintana* was obtained by using 50 mg (wet weight) of cells grown on selective agar medium. The DNA was purified using the small-scale preparation method (1).

DNA probes for Southern blot analysis were labeled with [³²P]CTP by primer extension (6), using a random-primer labeling kit from Dupont, NEN Research Products (Boston, Mass.).

Restriction enzymes were purchased from Bethesda Research Laboratories and used according to directions. Digested DNA was electrophoresed in 1% agarose (Ultra-Pure; Sigma Chemical Co., St. Louis, Mo.) in Tris-acetate buffer (13) for 7 h at a constant 35 mA. The DNA was transferred from the gels to nitrocellulose filters (Schleicher & Schuell, Inc., Keene, N.H.), and hybridization was carried out by using standard procedures (23). The blot was then washed four times with 0.1 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 68°C (to allow a 2% mismatch), dried at room temperature, and exposed to XAR5 film (Kodak, Rochester, N.Y.) at -70°C with two Cronex Lightning-Plus intensifying screens (Dupont NEN).

Plasmid construction. Since tetracycline is the drug of choice for many rickettsial infections, the gene for tetracycline resistance (Tet^r) was routinely inactivated or excised from all vectors. The plasmid pSUP204Ts was derived from pSUP204 (18) by excising the tetracycline resistance gene. This was accomplished by digesting the plasmid to completion with a mixture of *Clal* and *BalI*. The sticky ends were filled in, using Klenow fragment, and the resulting blunt ends were ligated with T4 ligase (13). The plasmid pUCK18, was constructed by first excising the kanamycin resistance gene (Kan^r) from pMB2190 (17) by using *EcoRI*. The Kan^r fragment was isolated by gel electrophoresis, and DNA was extracted with GeneClean (Bio 101, La Jolla, Calif.). The Kan^r gene was then ligated into a *EcoRI*-digested pUC18 (30).

The cosmid pEST is a truncated version of pHK17 (9, 10) from which Tet^r had been excised by a partial digestion with *HaeII* and then ligated with T4 ligase (22).

CAT assay. The method of Gorman et al. (7), was used to

TABLE 1. Effect of voltage on survival and transformation frequency of *R. quintana*

Voltage ^a (kV/cm)	Total % survival ^b	Transformants/ μ g of DNA ^c
12.5	20	3.09×10^5
10.0	32	1.35×10^4
8.0	36	2.75×10^3
6.0	31	2.16×10^2

^a Delivered as a 10-ms pulse.

^b Percentage of initial input ($4 \times 10^8/40 \mu$ l) of *R. quintana* surviving. Each percentage represents the average of at least three separate experiments. Immediately after electroporation, cells were transferred to 1 ml of broth growth medium and incubated for 5 h at 35°C. The cells were then plated on nonselective medium, and colonies were counted.

^c The vector used was pEST. Transformants were plated onto a selective medium containing 15 μ g of KAN per ml.

detect chloramphenicol acetyltransferase (CAT) activity. Briefly, cells were harvested and washed three times in phosphate-buffered saline and suspended in 100 μ l of 0.25 M Tris hydrochloride. The cell suspension was lysed by two cycles of freeze-thawing and centrifuged, and the supernatant was recovered. One microliter of [¹⁴C]CAM (57 mCi/mmol) (Dupont NEN), 20 μ l of 4 mM acetyl coenzyme A (Sigma) plus 70 μ l of 1 M Tris hydrochloride at pH 7.8 were added to 60 μ l of cell extract and incubated for 1 h at 37°C. CAM and its derivatives were extracted with 1 ml of ethyl acetate. The top phase was collected and evaporated to dryness in a vacuum oven at 37°C for 2 h. The dried material was suspended in 30 μ l of ethyl acetate, spotted onto Kodak 13181 silica gel thin layer chromatography sheets, placed in 95% chloroform-5% methanol solvent, and subjected to chromatography for 2 h. Autoradiograph exposures (for 16 h) were made with Kodak film at -70°C, using intensifying screens.

RESULTS

Effect of voltage on survival and transformation of *R. quintana*. To optimize conditions for entry of DNA into *R. quintana* by electroporation, a variety of voltage levels, pulse lengths, expression times, and incubation conditions were used. Previous studies with *E. coli* suggested that transformation efficiency is optimal when voltage and time conditions are adjusted to allow for survival of 25 to 50% of the initial cell population (5). Table 1 shows the effects of voltage on survival of *R. quintana*. It is apparent that as the voltage is reduced, more cells survive. At 12.5 kV/cm with a pulse time of 10 ms, approximately 20% of the cells survived. Conversely, when exposure was shortened to 5 ms, more than 35% survived (data not shown).

In preliminary experiments with *E. coli*, maximum transformation frequencies were obtained at 12.5 kV/cm and 5 ms. At these settings and with 250 μ g of DNA per ml, transformation efficiencies were as high as 98%. The efficiency of transformation decreased in proportion to decreasing field strength (data not shown). Based on this information and the survival data shown in Table 1, experiments with *R. quintana* were initially carried out over a range of voltages and at pulse lengths of 5 and 10 ms. Cells were plated either onto agar plates containing only growth medium (to determine viability) or onto agar plates containing growth medium and 15 or 20 μ g of KAN per ml (to select for transformed *R. quintana*). Untransformed organisms do not grow on plates containing 10 μ g of KAN per ml (15), but transformed colonies grew at 15 μ g/ml (Table 1). Essentially no difference

FIG. 1. Southern hybridization analysis of DNA from *R. quintana* probed with 32 P-labeled pEST DNA. The different DNAs were digested with *Hinc*II, and the DNA fragments were separated on a 1.0% agarose gel, transferred to nitrocellulose, and hybridized with 32 P-labeled pEST (labeled by random primer extension). Lane 1, pEST cosmid DNA; lane 2, DNA from untransformed *R. quintana* cells; lane 3, CsCl-purified total DNA from *R. quintana*; lane 4, DNA from transformed *R. quintana* isolate A; lane 5, DNA from transformed *R. quintana* isolate B; lane 6, DNA from transformed *R. quintana* isolate C. DNAs in lanes 2, 4, 5, and 6 were prepared by the method of Birnboim and Doly (1). kb, Kilobases.

in cell numbers was observed with the two KAN concentrations (data not shown). At 12.5 kV/cm exposure, with a pulse time of 10 ms, transformation efficiencies of approximately 0.3% with 20% viability and 3.1×10^5 transformants per μ g of cosmid DNA were obtained. At 5-ms exposure, viability increased to 35%; however, the number of transformants was reduced approximately 50% (data not shown). Because a pulse time of 10 ms consistently resulted in twice the number of transformants, the 5-ms pulse time was not used further in these studies.

Verification of transformation by Southern blot analysis. Confirmation of cosmid DNA uptake by *R. quintana* was made by Southern blot analysis (23). A number of isolated colonies growing on selective media were picked, spread on individual plates containing selective medium, and incubated until confluent growth was evident. The DNA obtained from all of the isolates examined appeared identical and three isolates are shown (Fig. 1), after they were digested with *Hinc*II and probed with linearized, 32 P-labeled pEST. Lane 1 contains pEST DNA, and lanes 4, 5, and 6 contain DNA from three transformed colonies of *R. quintana*. Lanes 2 and 3 contain DNA extracted from nontransformed *R. quintana*. It is clear that the transformed colonies contain pEST DNA.

To ensure that the transformed *R. quintana* colonies were not the result of contamination, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (11) and immunoblot analyses were performed to compare original frozen stocks obtained from the American Type Culture Collection with the transformed cultures. The protein profiles appeared to be identical, indicating that the transformed cultures were *R. quintana* (data not shown).

Transformation of *R. quintana* with different plasmids. *R. quintana* not only recognized the Kan^r gene promoter and transcribed the gene but also replicated the pEST cosmid DNA. A variety of plasmids were examined for their ability to transform *R. quintana* to determine whether other replicons were capable of replication in this organism. A partial list of plasmids that were evaluated is given in Table 2. In addition to pEST, the plasmid pSUP204Ts, was successfully used to transform *R. quintana*. Plasmid pSUP204Ts contains both the pMB1 and RSF1010 replicons and CAM resistance

TABLE 2. Partial list of cosmids and plasmids used in attempting to transform *R. quintana*

Cosmid or plasmid ^a	Anti-biotic	Transformation	Replicon(s)	Southern verification ^b
pEST	KAN ^c	+	RK2	+
pSUP204Ts	CAM ^d	+	pMB1 and RSF1010	+
pUCK18	KAN	—	pMB1	—
pMK20 ^e	KAN	—	ColE1	—
pML31 ^f	KAN	—	F	—
pACYC184Ts ^g	CAM	—	p15A	—

^a One microgram of DNA per 40 μ l was used with each attempt.

^b Total DNA was extracted from transformed cells and hybridized with the appropriate 32 P-labeled DNA.

^c KAN (15 μ g/ml) was added to growth medium.

^d CAM (1 or 2 μ g/ml) was added to growth medium.

^e Data taken from reference 9.

^f Data taken from reference 12.

^g Data taken from reference 3. The tetracycline resistance gene was inactivated by digestion of pACYC184 to completion with *Bgl*I and *Cl*aI. The resulting plasmid was ligated by blunt end ligation (13).

(Cam^r). The untransformed organism is very sensitive to CAM and growth is completely inhibited at a concentration of 1.0 μ g of CAM per ml (15). In contrast, the transformed organisms grew on medium containing 1 or 2 μ g of CAM per ml. Transformation was not observed with the following plasmids: pACYC184 (3), pUCK18, pMK20 (9), or pML31 (12).

R. quintana transformed with pSUP204Ts was examined to determine whether the CAM resistance observed was due to the expression of the Cam^r gene present on this plasmid. Cell extracts of transformed and untransformed *R. quintana* were assayed for the presence of CAT activity encoded by the Cam^r gene (Fig. 2). Lane 1 contains [14 C]CAM alone, and lane 3 contains extracts from untransformed *R. quintana*. It is evident that the [14 C]CAM remains at the origin in its unmodified form. Lanes 2 and 4 contain extracts from *E. coli* and *R. quintana*, respectively, that have been transformed with pSUP204Ts. In contrast to lane 3, the CAT activity present in these extracts has converted the [14 C]CAM to inactive acetylated forms.

DISCUSSION

These experiments clearly demonstrate the transformation of *R. quintana* and are the first to show that a member of the

FIG. 2. CAT assay of cell extracts of *R. quintana* transformed with pSUP204Ts containing the Cam^r gene. pSUP204Ts containing *R. quintana* isolates were grown on plates containing 2 μ g of CAM per ml for 4 days and washed off with rickettsia suspension medium. *E. coli* DH5(pSUP204Ts) cells were grown to stationary phase in 30 ml of LB broth (13) containing 100 μ g of CAM per ml (4). Lane 1, [14 C]CAM control; lane 2, *E. coli* DH5(pSUP204Ts); lane 3, *R. quintana* nontransformant grown on nonselective medium; lane 4, *R. quintana* transformed with pSUP204Ts. Not all the [14 C]CAM in lane 2 was acetylated.

family *Rickettsiaceae* can be transformed. The transformed *R. quintana* grew on medium containing levels of KAN or CAM which were well above those required to completely inhibit growth of untransformed *R. quintana*. DNA hybridization studies confirmed that the transformed *R. quintana* retained the plasmid intact and CAT assays confirmed that CAM resistance was due to the presence of CAT activity.

Untreated *R. quintana* form visible colonies in 5 days on plate-to-plate passage, while transformed organisms took up to 16 to 17 days on 15 µg of KAN per ml to form visible colonies. The slower growth of transformed *R. quintana* appears to be the result of cellular damage due to treatment before electroporation (trypsin, etc.) and the electroporation process itself, rather than the presence of the plasmid. Control cells prepared for electroporation and then electroporated but not transformed took up to 14 days to form visible colonies. On continued subculture, both control cells and transformed cells formed visible colonies within 5 days.

Organisms transformed with pEST or pSUP204Ts were relatively stable; when they were transferred to nonselective medium, the plasmid was still present after two successive transfers for pEST and three successive transfers for pSUP204Ts, as determined by Southern blots (data not shown).

The data shown in Table 2 indicate that at least two replicons, RSF1010 and RK2, can be used to transform *R. quintana*. Attempts to transform *R. quintana* with several other plasmids were not successful (Table 2). These plasmids contain several different origins of replication, and it is not certain why we were unable to effect transformation. It is possible that the pMB1, ColE1, F, and p15A origins of replication are not effective in *R. quintana*. The inability to transform *R. quintana* with pUCK18 is especially interesting, since pSUP204Ts also contains a pMB1 origin. We have recently been able to transform *R. quintana* with a plasmid derivative of RSF1010 (data not shown), and thus it is probable that replication of pSUP204Ts occurs as a result of the RSF1010 origin, rather than the pMB1 origin. Another possible explanation is that since *R. quintana* appears to contain some restriction endonuclease activity (data not shown), some plasmid or cosmid constructions are degraded by this activity. Limited restriction analyses of plasmids successfully used to transform *R. quintana* to date do not reveal any base modifications. It is possible that the DNA vectors were subjected to some alterations (e.g., methylations) within the *R. quintana* cells which have not been detected. This is an intriguing possibility, since such modified vectors may be useful in improving transformation frequencies in *R. quintana* as well as other members of the *Rickettsiaceae*.

The efficiency of transformation thus far obtained with *R. quintana* is quite low. It is clear that higher voltages are needed to obtain efficient transformation. For example, dropping the voltage from 12.5 to 10.0 resulted in a loss of over 1 log of transformants (Table 1). These findings are consistent with results obtained by other workers (2, 14). Because of the smaller size of all members of the *Rickettsiaceae*, a higher electric field may be required to obtain levels of transformation comparable with those of other bacteria (14).

Studies with *Campylobacter jejuni* have shown that the efficiency of transformation varies with the phase of growth as much as 10-fold over a 24-h period (14). *C. jejuni* was transformed more efficiently when electroporation was carried out with early to mid-exponential phase cells. Calvin and Hanawalt (2) have shown that while log-phase *E. coli* are

also transformed somewhat more efficiently, these cells are extremely sensitive to killing by electroporation. Preliminary studies with *R. quintana* suggest that extensive killing occurs with cells in the log phase of growth (at least 99.5% at 12 kV/cm with a 5-ms pulse), while less killing (~75% death) occurs with stationary-phase cells (data not shown). Growth phase should be considered when attempting transformation with other plasmids.

The transformation of a member of the *Rickettsiaceae* family has important implications for understanding the genetics of these organisms and for investigating their mechanisms for intracellular parasitism. For example, transformation techniques provide a means for detecting and characterizing virulence factors which allow *Rickettsia* cells to invade mammalian cells or metabolic factors which allow *Rochalimaea* cells to grow extracellularly. If *Rickettsia* species can be transformed using techniques similar to those used for transforming *R. quintana*, it may be possible to introduce metabolic capabilities that supplement or complement their genetic capabilities and allow them to grow axenically. Standard genetic procedures could then be used to investigate their biochemical pathways and characterize their virulence factors.

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