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TRANSFORMATION AND GENOMIC RESTRICTION MAPPING OF *ROCHALIMAEA* SPP.*

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Summary. - Transformation procedures using electroporation were established for *Rochalimaea quintana.* Several cosmid/plasmids possessing the RK2 or RSF1010 origin of replication were successfully inserted. Plasmid retention and replication were verified by antibiotic resistance and Southern blot analysis. The highest level of transformation was obtained at a voltage field strength of 12.5 kV/cm with a pulse time of 10 miliseconds. Transformation efficiency was low (0.3 %) with approximately 10^5 transformants/microgram of DNA. One construct, designated pAG10, reached sufficient levels in *R. quintana* to be isolated by density gradient centrifugation. Analysis of this plasmid after several cycles of growth in *R. quintana* revealed no obvious modifications. Physical maps of *Rochalimaea* spp. chromosomal DNA using pulse-field electrophoresis are being developed. Digestion of *R. vinsonii* chromosomal DNA with *Not*I or *Sfi*I resulted in three and one fragments, respectively. When *R. quintana* was digested in a similar manner, both *Not*I and *Sfi*I produced four fragments. Double digestion of *R. quintana* DNA with *Not*I and *Sfi*I yield seven fragments ranging in size from 11 to 925 kb. Summing the fragments indicate an approximate genome size of 2.1×10^6 bp for *R. vinsonii* and 1.7×10^6 bp for *R. quintana* chromosomal DNA.

Key words: Rochalimaea; Trench Fever; transformation; electroporation; rickettsia; plasmids; pulsed field gradient electrophoresis; restriction; genomic mapping

Introduction

The members of the family *Rickettsiacae* have been separated into three

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genera: *Rickettsia, Coxiella,* and *Rochalimaea* (Weiss and Moulder, 1984). With the exception of those species in the genus *Rochalimaea,* all are obligate intracellular bacteria. Both *Rochalimaea quintana* and *Rochalimaea vinsonii* can be cultivated axenically and grow in epicellular locations within their eukaryotic hosts (Baker, 1964; Weiss, 1982), as well as on enriched media (Weiss and Dasch, 1982). Members of this genus appear to be related to some species of the genus *Rickettsia* (Myers and Wisseman, 1980; Weiss *et al.,* 1982; Weisburg *et al.,* 1985). It was reasoned that procedures developed to successfully transform *Rochalimaea* spp., might be applied to the obligate intracellular bacteria of the genus *Rickettsia.*

The ability to transform rickettsiae would allow the introduction of specific mutations or genes and the phenotype observed. A technique of transformation using electroporation (Calvin and Hanawalt, 1988; Dower *et al.,* 1988; Miller *et al.,* 1988) has been developed to introduce DNA into *Rochalimaea.* Previous studies have demonstrated by plasmid encoded antibiotic resistance genes, that plasmid/cosmid DNA replicates and is expressed in *R. quintana* (Reschke *et al.,* 1990). The present report demonstrates the ability of the plasmid pAG10, possessing the RSF1010 origin of replication, to successfully transform and replicate in *R. quintana.*

In anticipation of locating specific genes within the genome of *R. quintana,* we have also begun work on obtaining a physical map using pulse-field gradient electrophoresis (PFGE) (Smith and Cantor, 1986). Using combinations of the rare cutting restriction endonucleases (RE), *Not*I, and *Sfi*I, a physical map of restriction sites within a genome can be established. A genetic linkage map would then be generated from these data by hybridizing cloned genes to these fragments (Southern, 1975), locating their positions relative to the various restriction sites.

Materials and Methods

DNA preparation and electroporation. R. quintana (Trench Fever) ATCC VR-358, Fuller strain, and *R. vinsonii* ATCC VR-152 Lot 6D, strain Baker, were obtained from the American Type Culture Collection, Rockville, MD. *Escherichia coli* DH5 and HB101 (BRL; Bethesda Research Laboratories Inc., Bethesda, MD.) were used to prepare cosmid and plasmid stocks by standard methods (Maniatis *et al.,* 1982). The growth and preparation of *R. quintana* for transformation has been previously described (Reschke *et al.,* 1990). DNA was suspended in sterile water to minimize the presence of electrolytes which can cause arcing and cell destruction (Dower *et al.,* 1988). Chromosomal DNA from *R. quintana* was prepared as previously described (Clewell and Helinski, 1972). Southern blot (Southern, 1975) and RE analysis of pAG10 DNA from *R. quintana* required ~500 mg (wet wt.) of cells from isolated clones grown on selective agar media. Cells were lysed with thermolysin and sodium dodecylsulphate at 37 °C and cesium chloride-ethidium bromide added (Reschke *et al.,* 1990). The lysate was centrifuged for 10 min at 10.000 rev/min to remove cellular debris. The material was then centrifuged at 45.000 rev/min for 48 hr in a Ti 60 fixed angle rotor and the plasmid band removed. The process was repeated and plasmid DNA was purified by ethanol precipitation (Calvin and Hanawalt, 1988).

Electroporation was performed using a Bio-Rad Gene Pulser unit set at voltage field strengths from 6-12.5 kV/cm, and at pulse times of 5 and 10 ms. The cells were removed from the cuvette, placed in 1 ml sterile broth medium (Weiss and Dasch, 1982), and incubated at 35 °C in 95 % air/5 % CO_2 at 100 % humidity without shaking. After 48 hr the cells were plated onto a selective medium and incubation continued. Transformed colonies were detected after 12 to 17 days.

Plasmid construction. pAG10 was derived from pKT212(Bagdasarian, et. al., 1979) by digesting with *Hind*III to excise the tetracycline resistance gene. It contains the RSF1010 origin of replication (Guerry *et al.*, 1974) and chloramphenicol resistance.

Pulse-field gradient electrophoresis (PFGE). The Bio-Rad CHEF-DR II electrophoresis system was used with the Bio-Rad model 200/2.0 constant voltage supply with the Bio-rad pulsewave™ 760 Switcher. All gels were 1 % BRL Ultrapure agarose in 0.5 x TBE buffer (100 mmol/l Tris pH 8.9, 100 mmol/l boric acid, 0.2 mmol/l EDTA) at 14 °C, 150-200 volts, and at 60 to 90 sec pulse times for various durations.

Results and Discussion

Transformation of R. quintana

Optimum transformation efficiencies are obtained at survival rates of 25-50 % (Dower *et al.*, 1988). As seen in Table 1 pulse times of 10 ms of various voltages and 5 ms at 12.5 kV/cm approximated this range of survival. Maximal transformation efficiency of 0.3 % was observed at 12.5 kV/cm and 10 ms (25μF and 400 ohms) with a viability of 20 %. This was equivalent to 3×10^5 transformants per μg DNA. At a 5 ms exposure, although there was an increase in viability (Table 1), the number of transformants was reduced by approximately 50 % (Reschke *et al.*, 1990).

Transformation using the plasmid pAG10 was demonstrated by growth on selective media and by Southern blot analysis (Southern, 1975). As seen in Fig. 1, a third lower band appears with the three transformed isolates and hybridizes with the pAG10 probe. This may be caused by de-methylation of RE sites. It is apparent that the plasmid is retained and that the RSF1010 replicator of pAG10

Table 1. Percent *Rochalimaea quintana* surviving electroporation

Voltage (kV/cm)	Pulse length (miliseconds) 5	10
12.5	35[a]	20
10.0	38	32
8.0	59	36
6.0	60	31

[a] Percent of initial input ($4 \times 10^8/40\mu$l) of *R. quintana*. Each percentage represents the mean of at least three experiments. After electroporation cells were transferred to 1 ml of broth medium and incubated for 5 hr at 35 °C. Cells were then plated on non-selective medium and colonies counted.

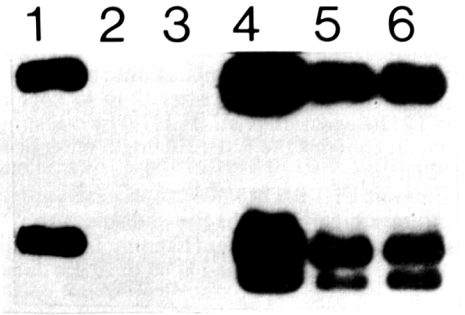

Fig. 1
Hybridization analysis of DNA from *R. quintana* transformants probed with ^{32}P-labelled pAG10 DNA
All DNAs were digested with *Pst*I. DNA fragments were separated on a 1.0 % agarose gel, transferred to nitrocellulose and hybridized with (^{32}P)-pAG10. Lane 1, pAG10 from *E. coli;* lane 2, DNA prepared by „mini-prep" technique (Birnboim and Doly, 1979) of untransformed *R. quintana;* lane 3. CsCl gradient purified chromosomal DNA of untransformed *R. quintana;* lanes 4-6, CsCl purified DNAs from individual transformed isolates. Stringency was 2 % mismatch.

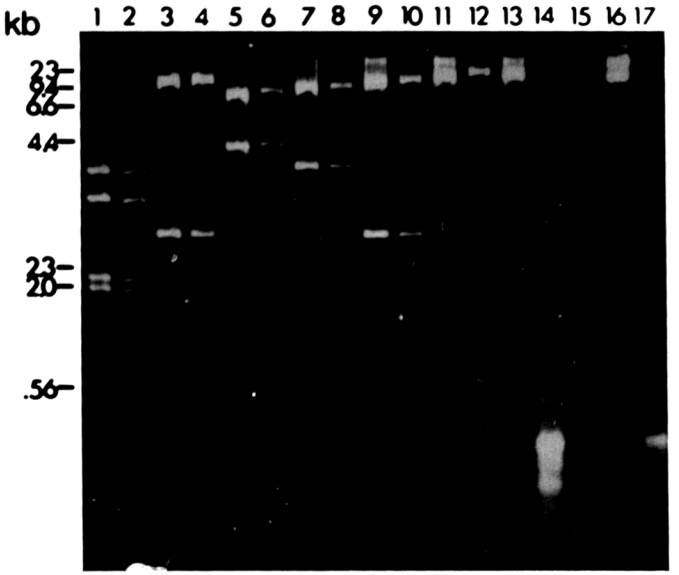

Fig. 2
Restriction analysis of pAG10 derived from *R. quintana*
Lanes 1 (Ec) and 2 (Rq) were digested with *Bgl*. An additional faint band appears at about 2.7 kb in lane 2. Lanes 3 (Ec) and 4 (Rq) were digested with *Hinc*II. Lanes 5 (Ec) and 6 (Rq) were digested with *Pvu*II. A third faint fragment appears at about 2.1 kb in lane 6. Lanes 7 (Ec) and 8 (Rq) were digested with *Pst*I. An additional faint third fragment appears at about 2.6 kb in lane 8. Lanes 9 (Ec) and 10 (Rq) were digested with *Stu*I. Lanes 11 (Ec) and 12 (Rq) were digested with *Xho*I (bands at about 200 bp are faint). Lane 13 is linearized pAG10. Lanes 14 (Ec) and 15 (Rq) were digested with *Sau*3AI. Lanes 16 (Ec) and 17 (Rq) were digested with *Mbo*I. Molecular size markers in kb indicated at right margin.

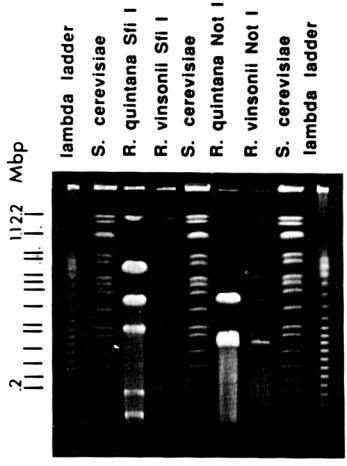

Fig. 3
High molecular size pulse-field gradient electrophoretic digestion fragments of *R. quintana* chromosomal DNA using the endonucleases *Not*I and *Sfi*I
1 % agarose in 0.5 x TBE buffer; 60 sec pulses for 15 hr followed by 90 sec pulses for 12 hr at 200 volts at 4 °C. *Not*I digested *R. quintana* chromosomal DNA into three fragments of sizes 702 kb, 493 kb, and 460 kb. The last two fragments are seen here as a single band. On other gels they are resolved into two bands. *Sfi*I digestion of *R. quintana* gives four fragments 925 kb, 560 kb, 147 kb, and 59 kb. The additional band seen at ~730 kb is assumed to be a partial digestion fragment (data not shown). *R. vinsonii* is digested into three fragments with *Not*I (850 kb, 730 kb, 530 kb), and not at all with *Sfi*I.

is recognized by *R. quintana*. The chloramphenicol resistance gene promoter is also recognized and the gene product is expressed.

Fig. 2 shows restriction enzyme analysis of pAG10 derived from *E. coli* and *R. quintana*. The anomalous bands associated with digested *R. quintana*-derived plasmid (lanes 2, 6, and 8 - see Figures) may be caused by de-methylation and subsequent cleavage of sites normally blocked in methylated *E. coli*. *Mbo*I and *Sau*3A are isoschizomers and are often used to assay methylation events. Although the *Sau*3A restriction digest of both *E. coli* and *R. quintana*-derived plasmid were identical (lanes 14, 15), *Mbo*I could only digest *R. quintana*-derived plasmid (lane 17) and not *E. coli*-derived pAG10 (lane 16), suggesting *R. quintana* removed the methyl group blocking proper cleavage. Such modified plasmids may increase the frequency of transformation of obligate intracellular rickettsiae in future experiments.

Physical mapping of Rochalimae spp.

It is now possible to construct physical maps of bacterial genomes using PFGE methods to separate the large DNA fragments produced when genomic DNA is digested with RE that recognize rare cutting sites. In this procedure intact cells are embedded in agarose, the cells are lysed *in situ,* and the released DNA cut with a RE that recognized rare sites.

Fig. 3 shows an example of a high molecular size RE digest of chromosomal DNA of *R. quintana* and *R. vinsonii*. A total molecular weight of 1.7×10^6 bp and 2.1×10^6 bp were obtained, respectively. *R. quintana* was digested into four fragments with either *Not*I, and *Sfi*I. *R. vinsonii* was digested into three fragments with *Not*I, and *Sfi*I did not cleave *R. vinsonii*. Table 2 summarizes the results of the pulse-field electrophoresis data to date. We have encountered

Table 2. Genomic restriction fragments of *Rochalimaea quintana* and *Rochalimaea vinsonii*

R. quintana – kbp			*R. vinsonii* – kbp	
*Sfi*I	*Not*I	*Sfi*I/*Not*I	*Sfi*I	*Not*I
925	740	(660)[c]	2 110	850
(730)[a]	460[b]	540		730
560	450[b]	480		530
147	18	430		2 110
59	1 668	147		
1 691		49		
		18		
		11		
		1 675		

[a] band due to incomplete digestion of fragments 560 and 147 and is not included in the sum.
[b] these two bands are not clearly resolved on PFGE and the band seen is assumed to be a doublet.
[c] band due to incomplete digestion of fragments 540 and 147 and is not included in the sum.

difficulties with partial digestion products. These difficulties are currently being resolved using higher concentrations of enzyme and by using other rare cutting RE. These fragments were transferred to nitrocellulose to be probed later with specific genes to locate their position relative to a fragment. From this data a rudimentary genetic map can eventually be generated.

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