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Isolation and Characterization of a Plasmid from Phase I *Coxiella burnetii*

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The DNA from the Nine Mile phase I strain of *Coxiella burnetii*, the etiological agent of Q fever, has been isolated and purified by cesium chloride-ethidium bromide density gradient centrifugation. A fraction of this DNA has a density characteristic of plasmid DNA. The plasmid DNA was cut with 20 different restriction endonucleases and shown to be a discrete entity. The plasmid, designated QpH1, is approximately 36 kilobases in size and has a molecular mass of 2.4×10^7 daltons. A partial restriction map of QpH1 has been constructed by using the restriction endonucleases *Sal*I, *Kpn*I, *Pst*I, and *Xba*I. QpH1 DNA radioactively labeled by nick translation was used to show that sequences similar to the plasmid are also present in the phase II antigenic variant of *C. burnetii*.

Coxiella burnetii, the etiological agent of Q fever, is a member of the family *Rickettsiaceae* (9). This obligate, intracellular parasite progresses through its replication cycle in the phagolysosome of the host cell (4). The organism not only survives in this harsh environment but replicates within an expanding vacuole, eventually leading to cell death. *C. burnetii* is the only rickettsia in which an antigenic phase variation has been demonstrated (25). Organisms isolated from natural infections are predominantly in phase I, whereas repeated passage through embryonated eggs results in a conversion to phase II (2). Although the two phases are morphologically indistinguishable, they have been shown to differ in surface chemical composition (1, 13) and in virulence (14). Furthermore, phase I *C. burnetii* parasites from nonimmunized guinea pigs are less susceptible to phagocytosis by polymorphonuclear leukocytes than are phase II organisms (3). Vaccines prepared from phase I organisms are more effective than preparations made from phase II (18). These observations suggest that phase transition may be an important factor in the pathogenic properties of this organism. Surface changes of other procaryotic organisms have been shown to be associated with virulence (16, 22).

Because the organism is difficult to culture, relatively few genetic studies have been undertaken with *C. burnetii*, especially with regard to the characterization of cellular nucleic acids. The guanine plus cytosine content of its DNA has been determined to be 43 to 45 mol% (21, 24). The size of the chromosomal DNA is about 10^9 daltons (17). No attempts to isolate plasmid DNA from this organism have been reported.

Plasmid DNA often carries genes for supplementary activities that allow the organisms to survive or to compete in specific ecological niches. Plasmids have also been shown to possess specific properties that contribute to pathogenicity (11). Phase change in *C. burnetii* may represent an example of such activity. Isolation and characterization of plasmid DNA from *C. burnetii* may therefore help in understanding the mechanisms of phase change or virulence, as well as any relationship between these two characteristics.

We report here the isolation of plasmid DNA from *C. burnetii*, Nine Mile strain, phase I. The plasmid is approximately 2.4×10^7 daltons and was mapped according to relative restriction enzyme cleavage sites. Four enzymes were used in both single- and double-digestion reactions. A probe was prepared from the purified phase I plasmid DNA by nick translation and was used to detect the presence of complementary sequences in phase II DNA.

MATERIALS AND METHODS

Rickettsiae. *C. burnetii* Nine Mile strains, phase I (307GP/1TC/1EP, clone 7) and phase II (90EP/1TC/4EP, clones 3 and 4) (29), were used in these studies. These plaque-purified strains were obtained from M. G. Peacock, National Institute of Allergy and Infectious Diseases, Rocky Mountain Laboratories, Hamilton, Mont.

Propagation and purification of rickettsiae. The organisms were inoculated into 6-day-old, embryonated, antibiotic-free, White Leghorn eggs (H and N Hatch-

ery, Redmond, Wash.). The infected yolk sacs of viable embryos were harvested 8 days after inoculation. Except where noted, the yolk sacs were not frozen, and the rickettsiae were purified immediately after the infected yolk sacs were harvested. Rickettsial suspensions were prepared from these infected yolk sacs by a series of differential centrifugations, as described previously (15), with the following modifications. A second Celite treatment was added to the procedure immediately after the initial Celite treatment to improve removal of host materials. After the second Celite treatment, phase I organisms were suspended in SP buffer (0.25 M sucrose, 0.14 M KCl, 0.01 M KPO_4, pH 7.2) containing 10 mM $MgCl_2$ at 4°C for 20 min. The rickettsial preparation was then centrifuged at 1,000 × *g* for 15 min, the pellet was discarded, and the suspension was filtered through a type AP20 Millipore microfilter (29). Phase II organisms were not treated with $MgCl_2$ but were filtered as described for phase I. These steps were added to aid in removing residual Celite and contaminating mitochondria (20). Several preparations were further purified by sucrose density centrifugation as described by Thompson et al. (27).

The purified preparations were pelleted, the pellets were suspended in 50 mM NaCl–20 mM Tris-hydrochloride (pH 9.1), and the dry weight of the organisms was estimated spectrophotometrically (29). This preparation was then used directly for DNA isolation.

DNA isolation and purification. Purified *C. burnetii* organisms (60 to 70 mg, dry weight) were suspended in 50 mM NaCl–20 mM Tris (pH 9.1) and incubated for 60 min at 37°C with 100 μg of thermolysin (10). Lysis was completed by adding sodium dodecyl sulfate (SDS) to a 1% final concentration. The plasmid DNA in this lysate was separated from chromosomal DNA by cesium chloride-ethidium bromide (CsCl-EtBr) density gradient centrifugation (5). To each milliliter of lysate were added 0.91 g of CsCl and 0.1 ml of EtBr (5 mg/ml in 10 mM Tris-hydrochloride–1 mM EDTA, pH 8.0). This mixture was centrifuged to equilibrium at 40,000 rpm for 40 h at 25°C in a Beckman 60 Ti rotor. The DNA bands were located by using long-wave UV light, and the individual bands were collected from the top with a 12-gauge cannula. The refractive index of these fractions was determined with a Zeiss refractometer (model 60469). EtBr was removed by extraction with isoamyl alcohol saturated with 5 M NaCl. The DNA samples were then extensively dialyzed against 10 mM Tris (pH 7.4)–1 mM EDTA at 4°C and precipitated with 3 volumes of absolute ethanol overnight at −20°C. The DNA was pelleted by centrifugation at −20°C for 45 min at 15,000 rpm in an SS34 rotor (Sorvall). The DNA was washed with 70% ethanol, repelleted, and suspended in a small volume of 10 mM Tris (pH 7.4)–1 mM EDTA (7).

Electrophoresis, restriction analysis, and transfer of DNA to nitrocellulose filters. DNA samples were analyzed electrophoretically in 0.9% agarose gels (Bio-Rad) prepared with Tris-borate buffer (19). Samples were run with 4 volumes of DNA (1 to 2 μg) plus 1 volume of tracking-dye mix (0.05% bromphenol blue, 20% Ficol 400, 1% SDS, and 100 μg of RNase [Worthington] per ml). Bacteriophage lambda DNA obtained from Bethesda Research Laboratories was cleaved with restriction endonuclease *Eco*RI or *Hin*dIII and used for size markers. Restriction enzymes were purchased from New England Biolabs or Bethesda Research Laboratories. Reaction conditions for digestions followed the suppliers' recommendations.

DNA for hybridization analysis was transferred to nitrocellulose sheets (BA 85; Schleicher & Schuell) as described by Thomashow et al. (26). After transfer the filters were rinsed briefly in 0.3 M NaCl plus 0.03 M sodium citrate (2× SSC), air dried, and heat treated at 65°C for 10 h, followed by heating for 2 h at 80°C in a vacuum drying oven.

Preparation of labeled QpH1, filter hybridization, and autoradiography. Plasmid DNA was labeled by nick translation as described by Thomashow et al. (26), using ^{32}P-labeled dCTP (600 Ci/mmol; New England Nuclear Corp.). After incubation, DNA was extracted with phenol-chloroform, and the radioactively labeled DNA was separated from unincorporated nucleotides by chromomatography on a column of Bio-Gel P-10. The DNA prepared in this manner contained 1 × 10^8 to 2 × 10^8 cpm/μg.

Prehybridization of DNA bound to nitrocellulose filters was carried out in 6× SSC and 10× Denhardt solution (8) for 10 h at 55°C. The filters were then incubated in a hybridization solution containing 10^6 cpm of the denatured DNA probe (100°C for 10 min) per ml as described by Thomashow et al. (26). Hybridization was for 24 h at 55°C. The blots were then washed four times for 20 min at 55°C in 3× SSC–0.2% SDS–5 mM EDTA. They were then washed for 20 min in 0.3× SSC–0.2% SDS–5 mM EDTA and finally for 20 min in 0.1× SSC–0.2% SDS–5 mM EDTA to remove unbound probe.

Autoradiography of the nitrocellulose filters was at −70°C for 4 h with Kodak XR-5 film and Du Pont Cronex Lightning-Plus intensifying screens.

RESULTS

Rickettsial lysis and plasmid isolation. To enhance our chances of isolating a plasmid from *C. burnetii*, a procedure was developed in which the number of manipulations required to lyse cells and purify plasmid DNA was minimized. Previous experience had shown that *C. burnetii* is difficult to lyse and is resistant to the action of lysozyme. However, treatment of purified organisms with thermolysin, a highly active, broad-spectrum proteolytic enzyme with no nuclease activity (10), followed by the addition of SDS, resulted in efficient lysis as evidenced by a rapid initial rise in viscosity which increased further as the mixture was gently agitated. After centrifugation of the cell lysate to equilibrium in CsCl-EtBr, two discrete bands were clearly evident with long-wavelength UV light. The lower band was present in much smaller quantity and stained less intensely than the upper band of chromosomal DNA. The density of the lower band was about 1.615 g/cm^3, and that of the upper band was 1.580 g/cm^3. This is consistent with the densities reported for plasmid and chromosomal DNA under these isolation conditions (6). In subsequent CsCl gradients, the lower band of DNA disappeared with repeated ma-

TABLE 1. Summary of cleavage characteristics of QpH1 plasmid DNA[a]

Enzyme	Recognition sequence (5′ → 3′)	No. of restriction fragments	Fragment size (base pairs)
*Sma*I	CCC ↓ GGG	0	
*Pst*I	CTGCA ↓ G	2	27,000; 8,500
*Sal*I	G ↓ TCGAC	2	30,000; 5,500
*Kpn*I	GGTAC ↓ C	2	22,000; 14,000
*Bgl*II	A ↓ GATCT	6	20,300; 7,200; 5,700; 1,100; 800; 650
*Xba*I	T ↓ CTAGA	3	22,000; 7,500; 6,500
*Xho*I	C ↓ TCGAG	4	
*Bst*EII	G ↓ GTNACC	3	
*Sst*I	GAGCT ↓ C	3	
*Bcl*I	T ↓ GATCA	4	
*Pvu*I	CGAT ↓ CG	4	
*Pvu*II	CAG ↓ CTC	4	
*Sac*II	CCGC ↓ GG	6	
*Eco*RV	GATAT ↓ C	6	
*Bam*HI	G ↓ CATCC	8	
*Hind*III	A ↓ AGCTT	10	
*Eco*RI	G ↓ AATTC	10	
*Hpa*II[b]	C ↓ CGG	12	
*Msp*I	C ↓ CGG	12	
*Hha*I	GCG ↓ C	15+	

[a] QpH1 was incubated with the different restriction endonucleases and the fragments separated by agarose gel electrophoresis.

[b] Methyl sensitive.

nipulation, and the DNA was recovered at the density of less dense linearized DNA. This was consistent with the premise that the lower band of DNA was a supercoiled plasmid that had been made linear by nicking. The putative plasmid DNA was electrophoresed on a 0.9% agarose gel and was found to migrate as a single band slightly behind the 22.5-kilobase (Kb) fragment of *Hin*dIII digests of λ DNA. This DNA fraction was designated QpH1 and considered by these preliminary criteria to be a plasmid.

Characterization and mapping of QpH1 by restriction endonuclease digestion. To begin determining cleavage characteristics and to estimate the size of QpH1, the plasmid was digested with 20 different endonucleases, and the fragments were separated by electrophoresis in agarose gels (Table 1). Sixteen of the 20 enzymes utilized recognize hexanucleotide sequences and were chosen in an attempt to identify enzymes that would cleave at a single site in the plasmid. This unique cleavage site(s) could then be used as a reference point to identify restriction sites of other enzymes. As can be seen in Table 1, none of the enzymes cut QpH1 at a single site, although *Pst*I, *Sal*I, and *Kpn*I each cut the plasmid at two sites. Digestion of QpH1 with *Sal*I consistently showed a 6.5-Kb fragment. The quantity varied with the different isolates, as seen by hybridization; however, it was always present in less than stoichiometric amounts. We have tentatively interpreted this to be the result of heterogeneous cleavage, possibly due to varied methylation or to the presence of a sequence of reduced specificity (rather than an additional mapping fragment). Therefore, we have not included this fragment in our mapping strategy.

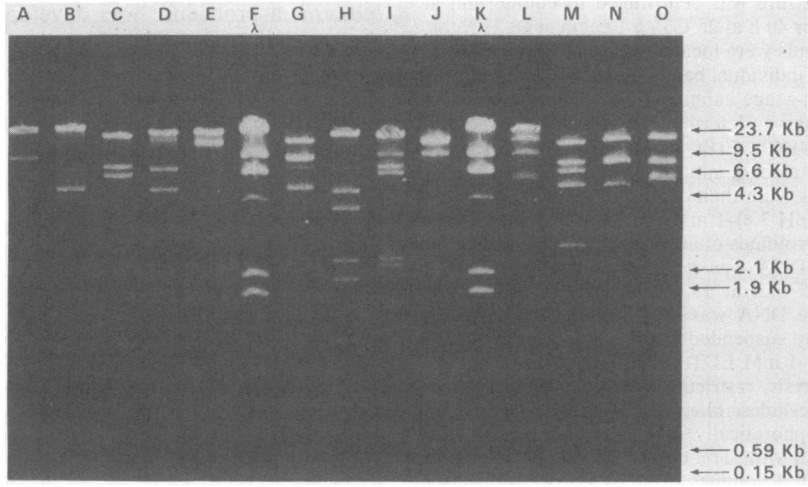

FIG. 1. Restriction endonuclease fragments of QpH1. QpH1 plasmid DNA was digested by the following enzymes and electrophoresed on 0.9% agarose gels (1 μg of DNA digested by 2 U of enzyme at 37°C for 3 h). Lanes: A, *Pst*I; B, *Sal*I; C, *Bgl*II; D, *Xba*I; E, *Kpn*I; F, λ phage DNA digested with *Hin*dIII; G, *Pst*I and *Sal*I; H, *Pst*I and *Bgl*II; I, *Pst*I and *Xba*I; J, *Pst*I and *Kpn*I; K, λ DNA digested with *Hin*dIII; L, *Kpn*I and *Sal*I; M, *Sal*I and *Bgl*II; N, *Xba*I and *Bgl*II; O, *Kpn*I and *Xba*I. Arrows indicate fragment size calculated from the λ phage standard.

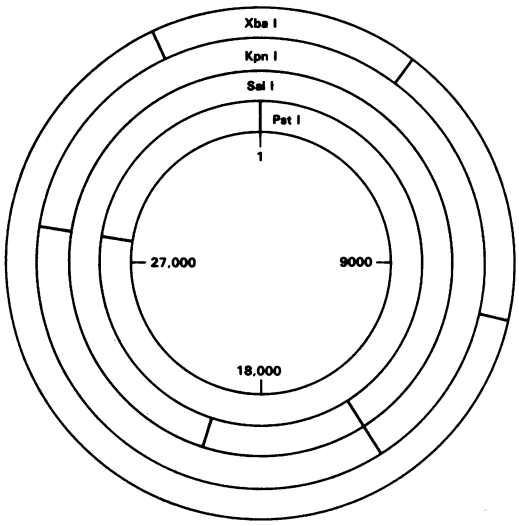

FIG. 2. Map of restriction endonuclease cutting sites. Approximate size of 36,000 base pairs. The *Pst*I site was chosen to represent base pair number 1.

Because cleavage with *Pst*I resulted in two well-separated and easily identified fragments, and since no unique cleavage site was detected in QpH1, restriction sites for *Sal*I, *Kpn*I, and *Xba*I (three fragments) were mapped relative to the two *Pst*I sites (Fig. 1). Measurement of the migration distances of the fragments generated by double and single digests with the four enzymes relative to size markers for *Hin*dIII digestion of λ DNA gave an estimate of the size of QpH1 at 36 Kb or 2.4×10^7 daltons.

Using the 36-Kb size estimate, the fragments generated in the single and double restriction digestions were arranged into the best fit, resulting in the restriction map shown in Fig. 2. Confirmation of this digestion map will require more definitive mapping techniques (i.e., cloning). Analysis of the fragments generated throughout the study suggests that only a single plasmid is present. The best preparations of rickettsial DNA resulted in a plasmid yield equivalent to 6.7% of the total DNA. Assuming a genome size of 10^9 daltons and a plasmid size of 2.4×10^7 daltons, a minimum copy number estimate of 3 can be calculated for the QpH1 plasmid.

Hybridization of QpH1 to phase I crude lysates. The isolation of QpH1 provided an opportunity to determine whether this plasmid or homologous sequences could be identified in phase II *C. burnetii*. This would allow direct evaluation of whether the presence or absence of these sequences was correlated with phase variation. Total DNA from crude lysates of two different clones of phase II, Nine Mile *C. burnetii*, as well as of phase I, Nine Mile *C. burnetii* linear DNA and QpH1 DNA, was digested with either *Eco*RI or *Sal*I. The resulting fragments were separated electrophoretically in agarose gels (Fig. 3). The fragments were transferred to nitrocellulose filters and hybridized with ^{32}P-labeled QpH1 (Fig.

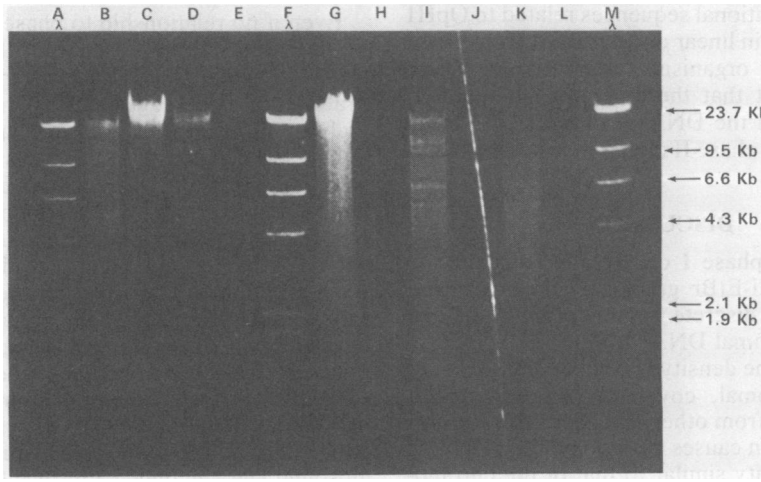

FIG. 3. Electrophoresis of phase II crude lysates for Southern blotting. Crude cell lysates were prepared from phase II *C. burnetii*. Total DNA was extracted with phenol-chloroform and digested with restriction endonucleases. Lane A, λ DNA digested with *Hin*dIII. Lanes B through E contained the following, digested with *Sal*I: B, phase I linear DNA fraction; C, phase II, clone 4 (fresh); D, phase II, clone 3 (frozen), E, QpH1; F, λ DNA digested with *Hin*dIII; lane G, phase I linear DNA fraction, undigested. Lanes H through K contained the following, digested with *Eco*RI: H, QpH1; I, phase II clone 3 (frozen); J, phase II clone 4 (fresh); K, phase I linear DNA fraction. Lane L, QpH1, undigested. Lane M, λ DNA digested with *Hin*dIII. Arrows indicate fragment size calculated from the λ phage standard.

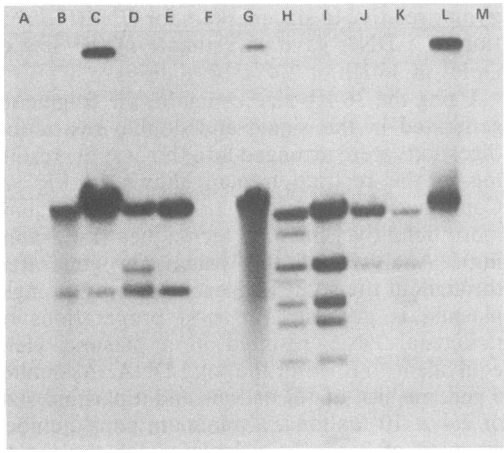

FIG. 4. Autoradiograph obtained by hybridization of radioactive QpH1 plasmid DNA with the Southern blot from Fig. 3. Lanes are as listed in the legend of Fig. 3.

4). All the fragments present in QpH1 digests (Fig. 4, lanes E and H) were also detected in linear DNA from phase I (Fig. 4, lanes B and K) and in the total DNA from the two phase II samples (Fig. 4, lanes C, D, I, and J). Presumably the presence of QpH1 sequences in phase I linear DNA resulted from nicking of the plasmid during DNA isolation. As in QpH1 plasmid DNA, the 6.5-Kb *Sal*I fragment was present in these other DNA preparations in variable and nonstoichiometric amounts relative to the other bands. No additional sequences related to QpH1 were detected in linear or total DNA from phase I or phase II organisms, respectively. These results suggest that there are no major differences between the DNA of phase I QpH1 and that of the two phase II clones digested with *Sal*I or *Eco*RI.

DISCUSSION

C. burnetii phase I cell lysates, analyzed in isopycnic CsCl-EtBr gradients and in agarose gels, contain a discrete fraction of DNA distinct from chromosomal DNA. In CsCl-EtBr isopycnic gradients the density is comparable to that of extrachromosomal, covalently closed circular plasmid DNA from other organisms (6). Repeated manipulation causes a portion of this DNA to assume a density similar to that of the chromosomal DNA. These observations indicate the presence of cryptic plasmid DNA in *C. burnetii*. Based on restriction enzyme mapping analysis, this DNA band appears to consist of a single plasmid, designated QpH1, of 2.4×10^7 daltons. This is considerably larger than the 10.8×10^6-dalton size reported for chicken mitochondrial DNA (28), which argues against the possibility that this DNA isolate is host contamination.

In an attempt to determine whether phase shift observed in *C. burnetii* is related to the loss or alteration of this plasmid, we used Southern blotting hybridization to probe for the presence of similar plasmid sequences in two Nine Mile, phase II clones. Based on restriction enzyme fragments detected by our plasmid probe, we find no major differences in the plasmid DNAs from the phase I and phase II variants. Therefore, phase variation does not result from a loss or gross alteration of the QpH1 plasmid.

These results do not necessarily eliminate the possibility that the plasmid has a role in phase variation. For example, a point mutation or an inversion of a small section of the plasmid DNA such as has been shown to occur in *Salmonella typhimurium* and *Neisseria gonorrhoeae* (16, 22, 23) would have escaped our detection unless it occurred in the cleavage sites for *Eco*RI or *Sal*I. Such inversions might be detected by incorporating additional restriction enzymes into the experimental strategy described here. Such an analysis would also help to determine whether insertion of the QpH1 into the host chromosome contributed to the observed phase transition. Another approach to analyzing whether QpH1 sequences are functionally different in phase I and phase II organisms would be to isolate and compare mRNAs from the two phases. Hybridization of labeled QpH1 to total RNA isolated from phase I and phase II *C. burnetii* organisms would reveal whether differential transcription of plasmid sequences was occurring.

Even if no relationship to phase variation can be determined, the presence of the plasmid in separately maintained strains after repeated laboratory culture suggests it is critical to cell viability (i.e., intracellular survival/replication). It may be that the plasmid codes for a protein which renders the cell envelope resistant to hydrolytic enzymes of the phagolysosome, as has been suggested for *Leishmania* (12). Alternatively, the plasmid might provide some metabolic function to capitalize on the unique substrate pool found in the phagolysosomal environment. Plasmids specifying entire metabolic pathways have been described in *Pseudomonas* spp. (30). The identification of a plasmid in this rickettsial agent thus provides a unique opportunity to probe the role of such extrachromosomal DNA in obligate intracellular bacteria.

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