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Characterization of the Origin of DNA Replication of the *Coxiella burnetii* Chromosome

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INTRODUCTION

Replication of the bacterial chromosome initiates at a unique site, the origin of replication (*oriC*), and proceeds bidirectionally.[1] DNA replication in bacteria is controlled at the level of initiation. Initiation of chromosome replication in the *Escherichia coli* cell cycle occurs when a certain cell mass per chromosomal origin of replication is obtained.[2] When a cell contains several origins, they are all initiated simultaneously.[3] The initiation of replication at *E. coli oriC* is a complicated event, which has been elucidated through construction of minichromosomes (plasmids which contain *oriC* as their only replication origin) and studies of minichromosome replication in an *in vitro* replication system. The chromosome replication origins of six species of gram-negative bacteria have been cloned and sequenced.[4-12] Included are members of the family enterobacteriaceae and of those distantly related to the enterobacteriaceae. The *oriC*s from organisms other than *E. coli* can function as origins in *E. coli* and use *E. coli* initiation factors. In comparing the nucleotide sequences of these bacterial *oriC*s, a consensus structure was found, which included multiple GATC sequences, DnaA binding sites, and AT-rich direct repeat regions.[12,13]

The minimal sequence necessary for autonomous replication of *E. coli oriC* is 245 bp.[14] However, DNA sequences adjacent to the right of this 245-bp minimal region are required for proper function of the origin *in vivo*. This sequence encodes a 16-kilodalton (kDa) MioC (modulation of initiation at *oriC*) protein of unknown function,[15] a 17-kDa AsnC protein, an activator of the *asnA* gene,[16,17]

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and the "incompatibility regions," *incB* and *incC*, which cover the *mioC* promoter and most of the *asnC* gene.[18,19] Transcription from the *mioC* promoter, negatively regulated by the DnaA protein *in vitro*[20] and *in vivo*,[21] is essential for proper origin function. Deletion of the nucleotide sequence covering the *mioC* promoter leads to decreased minichromosome copy number and increased segregational instability.[18,22,23]

Studies on minichromosomes have enabled us to characterize the replication properties of the *oriC* and to obtain information on the mechanisms of control of chromosome replication. Studies on *E. coli* minichromosomes containing *oriC* have indicated that (i) initiation of replication (on *E. coli* minichromosomes) requires a number of the same gene products as on the chromosome, including RNA polymerase, gyrase, primase, DnaA, DnaB, and DnaC proteins;[24–29] (ii) initiation of replication (in both systems) does not require the *polA* gene product, whereas colE1-type replicons do;[14,30] (iii) minichromosomes are present in higher copy numbers than the chromosome and are not maintained stably in the host;[18,21,31,32] and (iv) minichromosome replication frequency is governed by the same mechanism that controls chromosome replication.[33]

DNA replication of *Coxiella burnetii*, the obligate intraphagolysosomal bacterium, is not understood. Previous studies have shown that the microorganism replicated its chromosome and plasmid DNA independently after removal from eukaryotic host cells.[33a] The origin of the *C. burnetii* chromosome has been cloned by construction of the minichromosome using an ori-search plasmid (Chen *et al.*; manuscript in preparation). Three minichromosomes, pSYC1, pSYC2, and pSYC3, were obtained by this method. A putative origin was found in these minichromosomes, which was in a 5.8-kb *Eco*R I fragment and functioned as a replication origin in *E. coli* (Chen *et al.*; manuscript in preparation). As the microorganism is a gram-negative–like bacterium in many aspects,[34–37] similarities between the replication origins of *C. burnetii* and those of other gram-negative bacteria were expected. In the current study, we determined the homology of the origin of *C. burnetii* and those of other gram-negative bacteria by Southern hybridization analysis and determined some physiogenetic properties of the *C. burnetii* minichromosome in *E. coli*.

MATERIALS AND METHODS

Bacterial Strains, Plasmids and Growth Conditions

The bacterial strains and plasmids used are listed in TABLE 1. Bacteria were grown in L-broth[38] or on L agar plates at 37°C. Antibiotic-resistant cells were selected by addition of 50 μg/ml kanamycin (Calbiochem Corp., San Diego, CA), or 50 μg/ml ampicillin (Boehringer Mannheim Biochemicals, Indianapolis, IN) to the growth medium.

Preparation of Chromosomal and Plasmid DNAs

C. burnetii Nine Mile strain phase I clone 7 (CB9MIC7)[39] was cultured and isolated as described previously.[40] *C. burnetii* chromosomal DNA was extracted by a thermolysin-SDS procedure.[41] Chromosomal DNAs of the gram-negative bacteria listed in TABLE 1 were isolated by a procedure described by Takeda *et al.*,[8] except

that 0.1% SDS was used as a substitute for Sarkosyl. DNAs were then purified via cesium chloride–ethidium bromide gradient centrifugation.

Plasmid DNAs were isolated in small scale by an alkaline-lysis procedure,[42] and in large scale by a cleared lysate procedure[43] followed by cesium chloride–ethidium bromide equilibrium centrifugation.

Enzymes

Restriction endonucleases *Acc* I, *Ava* I, *Bam*H I, *Bgl* II, *Cla* I, *Eco*R I, *Eco*R V, *Hinc* II, *Hind* III, *Hpa* I, *Kpn* I, *Nci* I, *Pst* I, *Pvu* I, *Pvu* II, *Sal* I, *Sma* I,

TABLE 1. Bacterial Strains and Plasmids

Designation	Genotype[a] and Phenotype[b]	Source
E. coli strain		
JZ279	F⁻ recA56 hsdR lacY galK2 galT22 metB1 trpR55 supE44 supF58	J. Zyskind[5]
JZ294	F⁻ polA1 argH hsdR rpsL thyA36	J. Zyskind[5]
DH5αF′	F′ endA1 hsdR17 (r_k^-, m_k^-) supE44 thi-1 λ⁻ recA1 gyrA relA1 Δ(lacZYA-argF) U169φ80d lacZΔM15	BRL[c]
Salmonella typhimurium	Wild type	WVU[d]
Klebsiella pneumoniae	Wild type	WVU[d]
Enterobacter aerogenes	Wild type	WVU[d]
Plasmids		
pML21	ori(col) Km^r	J. Zyskind[44]
pJZ101	ori(col) ori(Eco) Ap^r	J. Zyskind[45]
pEMBL8 (+)	ori(col) ori(F1) Lac$^+$ Ap^r	L. Dente[46]
pSYC1	ori(Cbu) Km^r	This lab[e]
pSYC2	ori(Cbu) Km^r	This lab[e]
pSYC3	ori(Cbu) Km^r	This lab[e]

[a] Abbreviations used are those of Bachmann and Low.[47]

[b] Abbreviations for drug resistance: Ap, ampicillin; Km, kanamycin. The type of origin of DNA replication carried by plasmids is shown as col, colE1 replicon; Eco, *E. coli oriC*; F1, phage F1 replicon; Cbu, *C. burnetii oriC*.

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[e] Details of the construction of these three *C. burnetii* minichromosomes will be published elsewhere.

*Sau*3A I, *Xba* I, *Xho* I (Bethesda Research Laboratories, Gaithersburg, MD) were used as recommended by the supplier. T4 DNA ligase (New England Biolabs, Inc., Beverly, MA), alkaline phosphatase (Boehringer Mannheim Biochemicals), and DNA polymerase I (Klenow fragment, Boehringer Mannheim Biochemicals) were used under conditions recommended by the suppliers.

DNA Labeling

Restriction endonuclease DNA fragments were labeled by the following methods: (i) oligo-labeling,[48,49] (ii) nick translation,[50] and (iii) 3′-end labeling.[51] Oligo-

labeling was used to label DNA with [α-^{32}P]dCTP; labeling was performed as recommended by the supplier (Multiprime DNA Labeling System; Amersham Corp., Arlington Heights, IL). A nick-translation kit (Bethesda Research Laboratories) was used to label DNA with biotin-11-dUTP (Bethesda Research Laboratories). The reaction was carried out as recommended by the manufacturer. The biotin-labeled DNAs were separated from unincorporated nucleotides by ethanol precipitation. The 3' end of the 5.8-kb *Eco*R I *C. burnetii* origin fragment was labeled by using the Klenow fragment of *E. coli* DNA polymerase I. The DNA fragment was labeled with [α-^{32}P]dATP in a 25-μl filling-in reaction mixture containing 1 μg of sample DNA; 1 μM [α-^{32}P]dATP (sp. act., 5000 μCi/mmole); 20 μM dCTP, dGTP, and dTTP; and 1 unit of the Klenow fragment of DNA polymerase I. The reaction mixture was incubated at room temperature for 30 min. The ^{32}P-labeled DNA was separated from the unincorporated [^{32}P]dATP using a chromatography column (nick column; Phamacia LKB Biotechnology, Piscataway, NJ) and was precipitated with ethanol. The 3'-end–labeled DNA was then used for restriction analysis.

DNA-DNA Hybridization

DNA-DNA hybridizations using ^{32}P-labeled DNA probes were performed by the standard procedure.[52] Alternatively, the biotin-labeled DNAs were used as probes, and hybridizations were carried out at 42°C in the presence of formamide. Prehybridization and hybridization were performed in 50% (prehybridization) or 45% (hybridization) formamide, 5% dextran sulfate, and 100 to 200 μg/ml biotinylated probe at 42°C for 16–20 h as recommended by the manufacturer (Bethesda Research Laboratories). The nitrocellulose filters were washed under high- or low-stringency conditions. The hybridized biotinylated probes were detected as recommended by the supplier (BluGene kit, Bethesda Research Laboratories).

Relative Copy Number Determination

E. coli JZ279[5] containing plasmids pSYC1, pSYC2, pSYC3 (see TABLE I) or containing pML21[44] was inoculated from an overnight colony into a selective medium (L-broth containing 50 μg/ml kanamycin) and incubated in a 37°C shaker to a density of 300 Klett units. Plasmid DNAs were extracted from 1.5 ml of culture by the alkaline-lysis procedure. A 5.19-kb DNA fragment was used as internal reference. This reference DNA was added to the cultures to a concentration of 0.133 μg/ml before extraction. Plasmid DNAs were suspended in 10 μl of 10 mM Tris-HCl (pH 8.0), 1 mM EDTA buffer containing 100 μg/ml RNAse. One fifth of the volume of each DNA extract was used for endonuclease digestion and agarose gel electrophoresis to determine the relative amount of these plasmid DNAs.

Test for Plasmid Stability

E. coli JZ279 containing plasmid pSYC1 or pML21 was inoculated from an overnight colony on selective agar into a selective medium. The cells were then incubated at 37°C with constant shaking overnight. The overnight culture was diluted with fresh, prewarmed, non-selective medium and incubated in a shaker at

37°C. At intervals (every two generations), the samples of the cultures were diluted with fresh L-broth and plated on selective and non-selective agar plates. To obtain exponentially growing cells for at least 20 generations, the cultures were diluted at suitable intervals (every two generations) with fresh, prewarmed, non-selective medium. The fraction of plasmid-containing colonies were determined by measuring the bacterial number on non-selective and selective media.

RESULTS

Determination of Homology between E. coli oriC *and the* C. burnetii *Origin*

Southern hybridization was used to determine whether *C. burnetii* chromosomal DNA contains a region homologous to *E. coli oriC* (FIG. 1). A 300-bp DNA fragment derived from pJZ101[45] by double digestion with restriction endonucleases *Ava* I and *Hin*d III was used as a biotin-labeled probe. This DNA fragment contains *E. coli oriC* sequences at positions −45 to +245.[6,7] The minimum region of the *E. coli oriC* is located between positions +22 to +267 of the origin sequence. Chromosomal DNAs from various gram-negative bacteria were used as reference controls. Sequences of the DNA in the *oriC*s of these bacteria[5,10] were 80% (or more) homologous to *E. coli oriC*.[11] Strong hybridization occurred to the origin fragment of these bacterial chromosomal DNAs (FIG. 1B): the 19.4-kb *Eco*R I fragment of *Salmonella typhimurium* DNA (lane 4),[9] the 17.5-kb *Sal* I fragment of *Enterobacter aerogenes* DNA (lane 5),[5] the 10.2-kb *Sal* I fragment of *Klebsiella pneumoniae* DNA (lane 6),[5] and the 9.4-kb *Eco*R I fragment of *E. coli* DNA (lane 7).[7] Very little hybridization to a 5–6-kb fragment of *C. burnetii* chromosomal DNA occurred (lane 3). No hybridization of the *oriC* to other bacterial chromosomal DNAs was observed when the same type of analyses was performed under high stringency conditions (not shown). The result indicated that the *C. burnetii* chromosome contained a 5–6-kb *Eco*R I fragment that had less than 80% homology to the *E. coli oriC*.

DNA homology of the origin region of the *C. burnetii* chromosome to those of other gram-negative bacterial chromosomes was also determined by Southern blot (FIG. 2). The 5.8-kb *Eco*R I *C. burnetii* origin fragment, derived from pSYC1 (Chen *et al.*, manuscript in preparation), was used as a ^{32}P-labeled probe (FIG. 2). Hybridization analysis was carried out under low stringency conditions, which allowed 25% mismatches. The probe hybridized only to *C. burnetii* DNA of the corresponding size (FIG. 2B). No hybridization to other bacterial DNAs was observed.

Restriction Map of the 5.8-kb C. burnetii *Origin Fragment*

Minichromosomes containing the *C. burnetii* origin as the only replication origin are not stably maintained in *E. coli* hosts (see below). Plasmid pSYC101, which has a higher copy number and which can be maintained stably in the host, was therefore constructed for the restriction map analysis. The 5.8-kb origin fragment obtained from *Eco*R I digestion of plasmid pSYC1 was ligated to an *Eco*R I–treated vector, pEMBL8(+).[46] *E. coli* DH5αF′ cells were transformed and used as the source of the 5.8-kb *C. burnetii* origin fragment. The restriction map (FIG. 3) of the *C. burnetii* origin fragment was deduced from molecular weights of restriction fragments obtained from gel electrophoresis of single, dou-

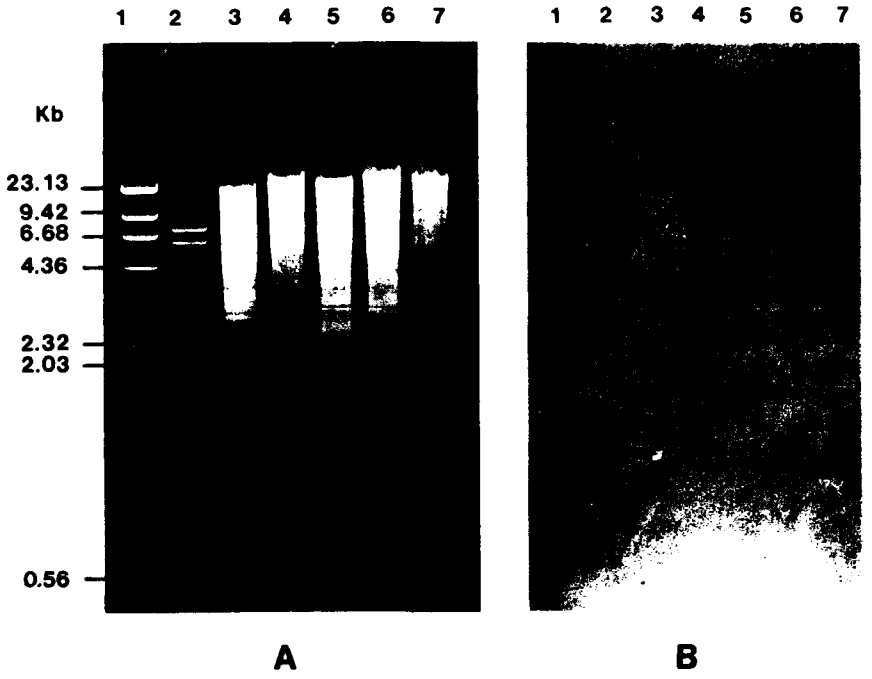

FIGURE 1. Southern blot hybridization of bacterial chromosomal DNAs to the *E. coli oriC*. **(A)** Ethidium-bromide staining of DNAs. **(B)** Southern blot hybridization using *E. coli oriC* as probe. Plasmid pSYC1 and chromosomal DNA from *C. burnetii, E. coli, S. typhimurium, E. aerogenes* and *K. pneumoniae* were digested with appropriate restriction enzymes. The restriction fragments were separated on a 0.9% agarose gel, transferred to a nitrocellulose filter, and hybridized to the biotin-labeled *E. coli oriC* probe (see text) as described in MATERIALS AND METHODS. The filter was washed under low stringency conditions. The hybridized biotinylated probes were detected by a BluGene technique as described in MATERIALS AND METHODS. **(Lane 1)** biotinylated *Hind* III fragments of lambda DNA, **(lane 2)** *Eco*R I digest of plasmid pSYC1, **(lane 3)** *Eco*R I digest of *C. burnetii* Nine Mile I clone 7 chromosomal DNA, **(lane 4)** *Eco*R I digest of *S. typhimurium* DNA, **(lane 5)** *Sal* I digest of *E. aerogenes* DNA, **(lane 6)** *Sal* I digest of *K. pneumoniae* DNA, **(lane 7)** *Eco*R I digest of *E. coli* DNA. *Arrowhead*, 5-6-kb fragment of *C. burnetii* chromosomal DNA shows little hybridization with the *oriC* probe.

ble, or partial digests of the DNA fragments with 18 restriction endonucleases (see below). 3'-end–labeled DNA was used in the partial digestion analysis. The following restriction endonucleases which recognize hexamers were used to map this DNA fragment: *Acc* I, *Ava* I, *Bam*H I, *Bgl* II, *Cla* I, *Eco*R I, *Eco*R V, *Hinc* II, *Hind* III, *Hpa* I, *Kpn* I, *Pst* I, *Pvu* I, *Pvu* II, *Sal* I, *Sma* I, *Xba* I, and *Xho* I. Those not appearing on the map (FIG. 3) have no recognition sites on this DNA fragment.

Properties of Plasmids Containing the C. burnetii *Origin of Replication*

DNA replication of colE1-type replicons requires *E. coli* DNA polymerase I, the *polA* gene product.[30] However, most gram-negative bacterial *oriC*s and plasmid origins other than the colE1 type do not require DNA polymerase I to initiate DNA replication. A transformation study using the *E. coli polA*$^-$ strain JZ294[5] as recipient host was done to determine the capability of the *C. burnetii* origin to function in the absence of DNA polymerase I. Transformation was carried out by the standard calcium chloride procedure.[53] Plasmid pSYC101, carrying both a colE1-type replicon and the origin of the *C. burnetii* chromosome, is able to transfer *E. coli* JZ294 to Km^r (data not shown). This indicated that (i) the *C.*

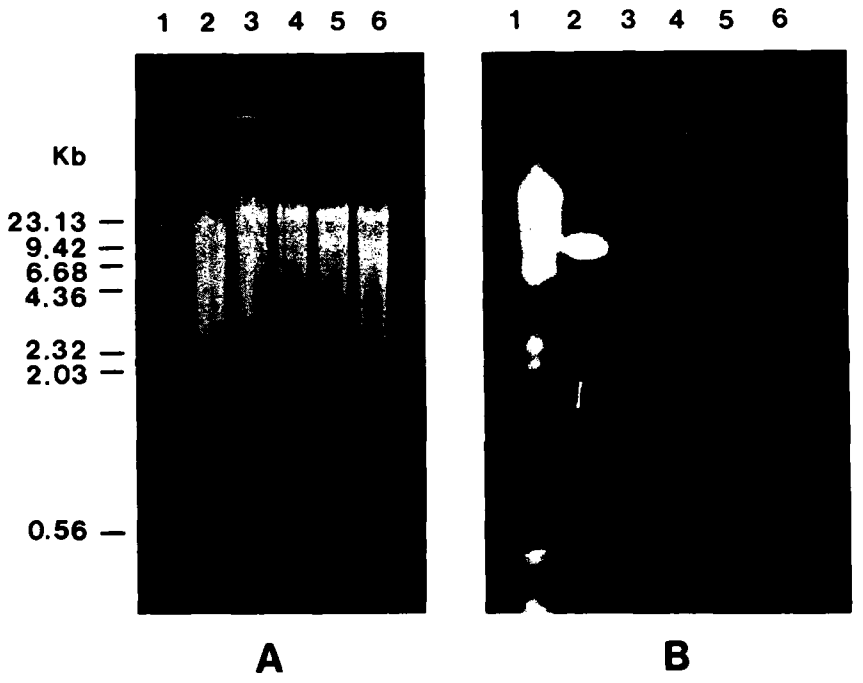

FIGURE 2. Southern blot hybridization of bacterial chromosomal DNA to the *C. burnetii* origin of DNA replication. **(A)** Ethidium-bromide staining of DNAs. **(B)** Southern blot hybridization using the ^{32}P-labeled *C. burnetii* origin DNA fragment as probe. Digested, electrophoresed DNAs were transferred to nylon filters (Hybond-N, Amersham Corp.) and hybridized to the ^{32}P-labeled probes (see text). The specific radioactivity of the probe was 2×10^8 cpm/μg. Hybridization was performed using 1×10^6 cpm of probe in the presence of sheared salmon sperm DNA. The membrane was washed under low stringency conditions. Autoradiographic exposure was for 20 h. **(Lane 1)** ^{32}P-labeled *Hin*d III fragments of lambda DNA, **(lane 2)** *Eco*R I digest of *C. burnetii* DNA, **(lane 3)** *Eco*R I digest of *E. coli* DNA, **(lane 4)** *Eco*R I digest of *S. typhimurium* DNA, **(lane 5)** *Sal* I digest of *K. pneumoniae* DNA, **(lane 6)** *Sal* I digest of *E. aerogenes* DNA.

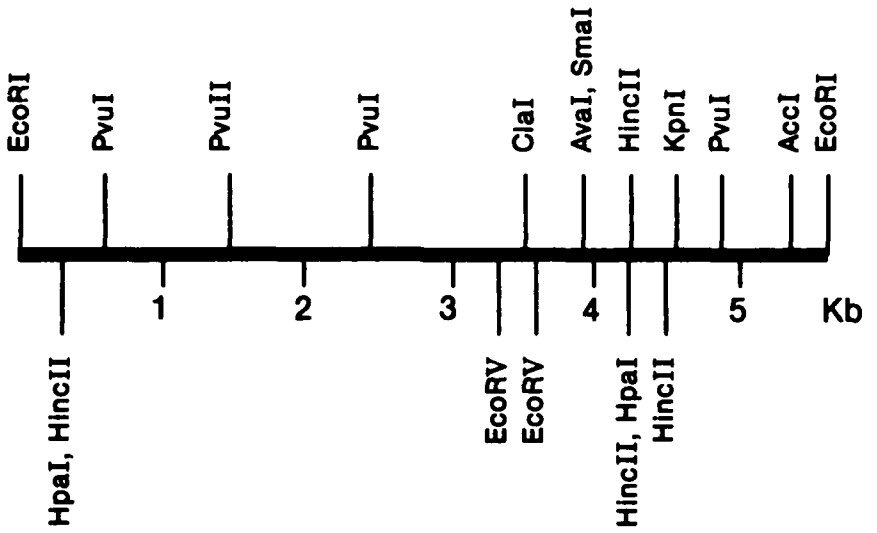

FIGURE 3. Restriction endonuclease recognition sites in the origin region of the *C. burnetii* chromosome.

burnetii origin was used for initiation of plasmid pSYC101 replication in the *polA*$^-$ strain and (ii) like most gram-negative bacterial *oriC*s, *C. burnetii oriC* does not require DNA polymerase I to initiate replication. The *polA*-independence of the *C. burnetii* origin made it possible to determine the minimal *oriC* region by an ori-test.[14]

The relative copy numbers of the minichromosomes containing a *C. burnetii* origin in *E. coli* JZ279 cells were determined by comparison of the density of DNA bands to those of pML21 in an ethidium bromide–stained gel (FIG. 4). These plasmids all contain a 6.8-kb kanamycin fragment, while all of the minichromosomes contain the 5.8-kb *C. burnetii* origin fragment. The marker DNA was used as internal reference control, which appeared equally in all of the samples. The amounts of plasmids pSYC1, pSYC2, and pSYC3 are about one-half or less of that of pML21.

The stability of *C. burnetii* minichromosomes in *E. coli* was determined by measuring the percentage of kanamycin-resistant cells after incubation in non-selective medium. Plasmid pSYC1 was lost rapidly during cell growth when selective pressure was removed (FIG. 5). However, plasmid pML21 remained relatively stable under the same test conditions. Statistically, the variation of the percentage of km^r cells containing pML21 is not significant (not shown). Percentages of km^r cells in the culture containing the *C. burnetii* minichromosomes dropped from 50 to almost 0 during growth in the non-selective medium for 20 generations, an interval of about 40 h (FIG. 5). About 40% of the overnight culture in selective medium consisted of plasmid-free cells (generation 0). This data suggested that the *C. burnetii* minichromosome was unstable in the *E. coli* cells, regardless of whether the selective pressure was present or not.

DISCUSSION

Hybridization analysis and the deduced restriction map of the *C. burnetii* origin suggested that the *C. burnetii* origin is not similar to the *E. coli* origin of replication, at least at the level of the primary structure. The nucleotide sequences of the *oriC* of *E. coli* and those of the reference bacteria used in this study are highly conserved (80–84% homology).[11] Strong hybridizations occurred between the *E. coli oriC* probe and the reference bacterial origin fragments under

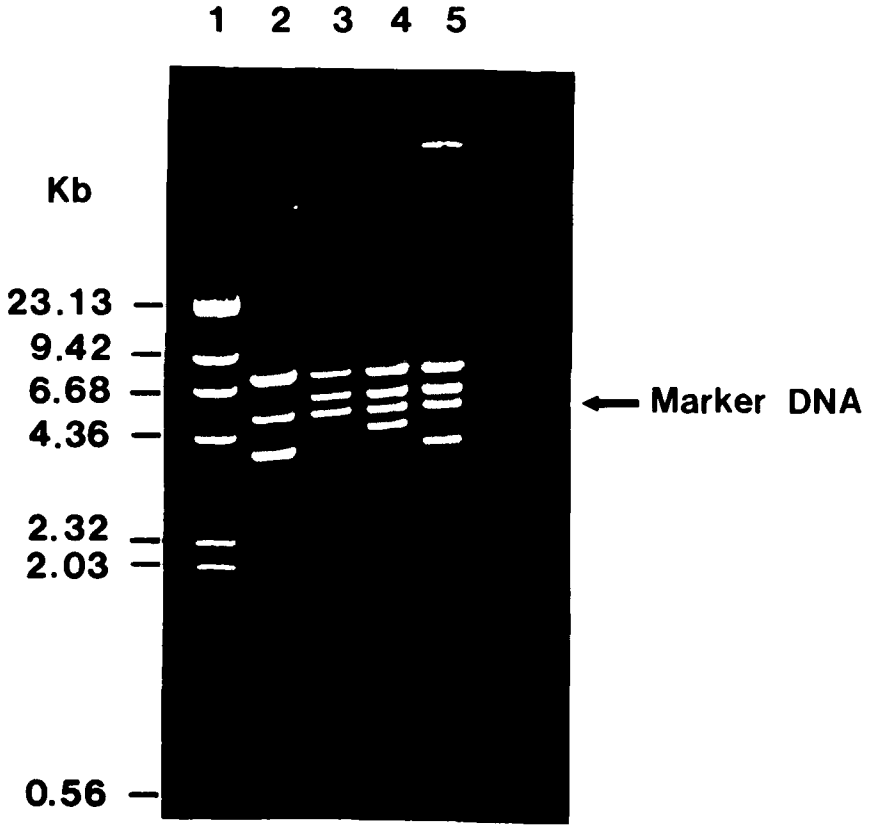

FIGURE 4. Gel electrophoretic determination of relative copy numbers of the Km^r plasmids containing the *C. burnetii* origin in *E. coli* JZ279. Cells were inoculated from an overnight colony in selective agar plates and were cultured in selective medium at 37°C to a density of 300 Klett units. After addition of 0.2 μg of the 5.19-kb DNA fragment as an internal reference, plasmid DNAs of each culture were isolated from 1.5 ml of culture. The relative amount of plasmid DNA was estimated by agarose gel electrophoresis. DNA was dissolved in 10 μl of 10 m*M* Tris-HCl, 1 m*M* EDTA buffer (pH 8.0) containing 100 μg/ml RNase. 2 μl of each preparation was used for *Eco*R I digestion and agarose gel electrophoresis. **(Lane 1)** size marker: *Hin*d III fragment of lambda DNA, **(lane 2)** pML21, **(lane 3)** pSYC1, **(lane 4)** pSYC2, **(lane 5)** pSYC3.

low stringency conditions (FIG. 1). However, hybridization between *E. coli oriC* and the *C. burnetii* origin fragment was much weaker. It is concluded that the *C. burnetii oriC* is less than 80% homologous to the *E. coli oriC*.

When the 5.8-kb *C. burnetii* origin fragment was used as probe, hybridization occurred only to the *C. burnetii* chromosomal DNA fragment with the corresponding length (5.8 kb: FIG. 2). The result was different from that in FIGURE 1: no hybridization was observed between *E. coli* and *C. burnetii* DNAs. This discrepancy might be due to the differences of both systems in the sensitivity of the probes, the method to detect the labeled probes, and the conditions of hybridization and post-hybridization wash. In any case, it demonstrates that there is very

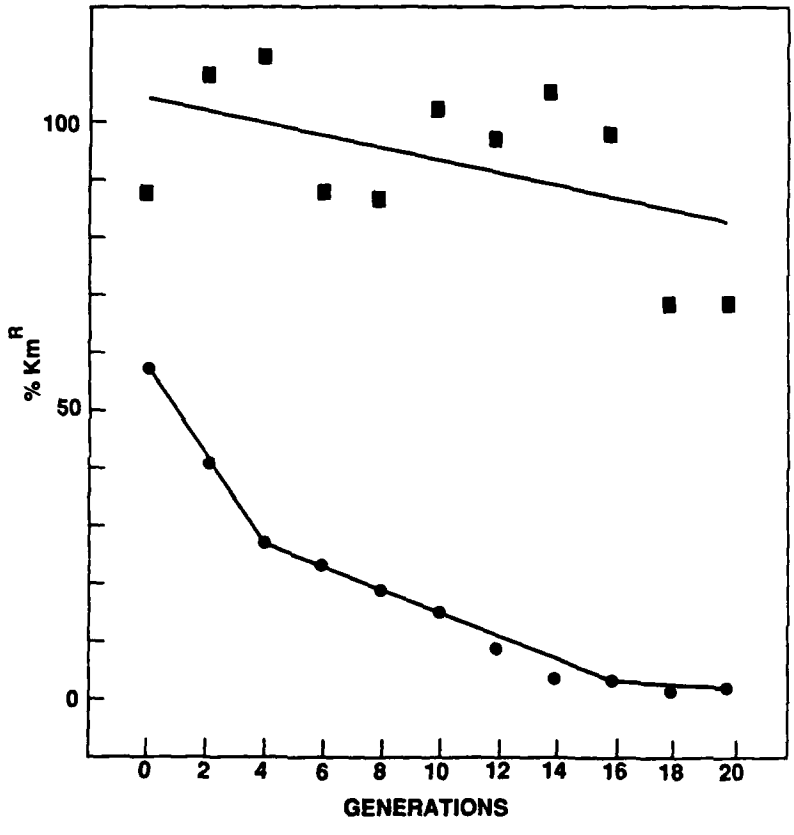

FIGURE 5. Stability of plasmid pSYC1 in *E. coli* JZ279. *E. coli* JZ279 containing the pSYC1 (●) or pML21 (■) was grown exponentially for 20 generations in non-selective medium as described in MATERIALS AND METHODS, and the samples were taken at suitable intervals. Percentages of plasmid-containing cells (kanamycin resistant, Km^R) were determined by direct counting of cells plated on selective and non-selective plates.

little DNA sequence homology between the origins of the *C. burnetii* chromosome and those of gram-negative bacterial DNA. The result also suggested that the regions adjacent to the *C. burnetii oriC* are not similar to those of *E. coli* or other tested gram-negative bacteria. The uniqueness of the *oriC*, and of its adjacent regions in the *C. burnetii* chromosome, is also implied by its restriction pattern. Restriction recognition sites, such as *Bam*H I, *Bgl* II, and *Pst* I, which appear one or more times in the tested gram-negative bacteria origins,[4,6,9,10] were not found in the *C. burnetii* origin fragment. Moreover, gene organization near the origin region of those gram-negative bacteria are similar.[5,9,10,54] The organization of open reading frames (ORFs) near the origin of the chromosome of the gram-positive bacteria *Bacillus subtilis* is similar to that at a location about 40 kb away from the *oriC* of the *E. coli* chromosome (*rpmH-dnaA-dnaN-recF-gyrB*).[55,56] The gene organization near the *B. subtilis* origin is believed to represent a primordial replicon from which the chromosome of both gram-positive and gram-negative bacteria have evolved. Further studies are needed to reveal the gene organization near the *C. burnetii oriC* and the evolutionary implications for the microorganism.

The properties of instability and high copy number are present in minichromosomes carrying *E. coli oriC* or other gram-negative bacterial origins.[5,9,18,21,31,32] Although the exact copy number of the *C. burnetii* minichromosome was not determined, comparison of its relative copy number to that of pML21 suggests that the *C. burnetii* minichromosome has only about one-half the copy number of pML21, which suggests that (i) *C. burnetii* minichromosomes have lower copy numbers than the colE1-type plasmids in *E. coli* but have a higher copy number than that of the *E. coli* chromosome or (ii) these minichromosomes are not stably maintained or segregated in the *E. coli* host. The decrease of the amount of plasmid DNA in non-selectively growth culture and the high proportion of plasmid-free cells in the growth culture (FIG. 5) reflected the instability of the plasmid. Moreover, the growth-curve studies indicate that cultures of *E. coli* JZ279 carrying the pSYC1 plasmid exhibited longer generation times than that carrying the pML21 plasmid (data not shown). This may be again due to the low percentage of the km^r cells in the whole population. *E. coli* minichromosomes initiate their replication in synchrony with the host chromosome.[33] Like the chromosome, minichromosomes initiate, on the average, at the same cell mass per origin;[2] and at a low growth rate the minichromosome was lost at a higher frequency.[21] The high loss-frequencies of minichromosomes might be an indication of (i) slight incompatibility, (ii) unstable segregation, or (iii) the presence of a high copy lethal (HCL) region. The incompatibility properties of *E. coli* minichromosomes have been shown to be in the *mioC* promoter, which was negatively regulated by DnaA protein and required methylation of the GATC site within the promoter sequence for full activity.[19] However, the *E. coli* minichromosomes can be stabilized by the *sop* (stability of plasmid) genes from plasmid F,[21] whose only function is proposed to be partitioning of plasmids at cell division. This fact indicates that the instability of *E. coli* minichromosome might be largely due to occasional segregation failure rather than to competition with the chromosome for replication factors. Another interpretation of the instability of the minichromosome is that it indicates the presence of a gene product which is detrimental to the host cell when present in higher than normal amounts.[5,9] Like *E. coli* minichromosomes, the instability of *C. burnetii* minichromosomes in *E. coli* hosts might be largely due to the lack of a partition system at cell division. The presence of a HCL region is less likely in the 5.8-kb *Eco*R I fragment, since pSYC101 is present in higher copy number in the *E. coli* host (data not shown).

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