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Cloning and Characterization of an Autonomous Replication Sequence from *Coxiella burnetii*

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A *Coxiella burnetii* chromosomal fragment capable of functioning as an origin for the replication of a kanamycin resistance (Kan^r) plasmid was isolated by use of origin search methods utilizing an *Escherichia coli* host. The 5.8-kb fragment was subcloned into phagemid vectors and was deleted progressively by an exonuclease III-S1 technique. Plasmids containing progressively shorter DNA fragments were then tested for their capability to support replication by transformation of an *E. coli polA* strain. A minimal autonomous replication sequence (ARS) was delimited to 403 bp. Sequencing of the entire 5.8-kb region revealed that the minimal ARS contained two consensus DnaA boxes, three A+T-rich 21-mers, a transcriptional promoter leading rightwards, and potential integration host factor and factor of inversion stimulation binding sites. Database comparisons of deduced amino acid sequences revealed that open reading frames located around the ARS were homologous to genes often, but not always, found near bacterial chromosomal origins; these included identities with *rpmH* and *rnpA* in *E. coli* and identities with the 9K protein and 60K membrane protein in *E. coli* and *Pseudomonas* species. These and direct hybridization data suggested that the ARS was chromosomal and not associated with the resident plasmid QpH1. Two-dimensional agarose gel electrophoresis did not reveal the presence of initiating intermediates, indicating that the ARS did not initiate chromosome replication during laboratory growth of *C. burnetii*.

Coxiella burnetii is the agent of Q fever in humans (65, 66, 69). The organism is an obligate bacterial parasite which replicates within acidic phagolysosomal vacuoles in eucaryotic host cells (1, 5, 6). During acid activation in vitro, the organism performs catabolic and anabolic reactions, including the synthesis of macromolecules (8, 19, 20, 75). Although growth has not been observed under these axenic conditions, the extent of metabolic activity, and especially of protein synthesis and DNA synthesis, can be remarkable (8, 20, 75). *C. burnetii*'s genome is 43% G+C (62), approximately 1.7×10^6 bp (46), and includes a 36×10^3-bp plasmid, QpH1 (52). Both plasmid and chromosomal DNAs are synthesized during acid activation (8). If replication from an origin were occurring in vitro, it would suggest that defined culture conditions for growth were near optimal. However, there were no means by which to unequivocally recognize initiation events in *C. burnetii*. The primary objective of the present research was, therefore, to clone and characterize the *C. burnetii* origin of replication (*oriC*). Because no natural or artificial transformation methods have been described for *C. burnetii*, information on origins or replication sequences took on secondary interest as well.

In *Escherichia coli*, initiation of DNA synthesis begins with the binding of multiple copies of the *dnaA* gene protein to sites within duplex DNA (3, 16). This aids strand separation. Helicase (the *dnaB* gene product) is guided onto the single strands at a rate of one molecule per replication fork. Hence, origins are characterized by several sites for *dnaA* gene protein binding and by having sites where helicase can be directed (perhaps by DnaC) onto a leading strand. The DnaA binding sites are characterized by the sequence 5′-TTATCCACA-3′ (16), and the helicase sites by A+T-rich direct repeats spaced within 5 to 20 nucleotides of each other and existing therefore as repeat 13-mers (or 16-mers). Several kinds of strategies are employed by various origins to assist each of these steps. For example, in *E. coli* other factors, such as integration host factor (IHF) or factor of inversion stimulation (FIS), may bind to specific sites and thereby assist in the intermediate phases of strand separation at origins (17, 49). The collective presence of DnaA boxes, A+T-rich regions, and FIS and IHF recognition elements (among others) thus make up indirect evidence for origin function. Alternatively, origin search can be conducted by hybridization screens that identify genes thought to characterize origin regions. Genetic loci that are often found near origins include *dnaA*, *dnaN*, *rpmH*, and *rnpA*; two uncharacterized proteins, termed the 9K protein and the 60K inner membrane protein, are also frequently found near *oriC*s. However, important exceptions exist. For example, the *E. coli* site most often utilized as an origin is located between *gidB* and *mioC* (42, 48, 57, 71); the sequence between *dnaA* and *rpmH*, which contains two DnaA boxes within the promoter of *dnaA*, is not known to function as an origin. Furthermore, there are multiple regions in some bacterial chromosomes that show origin character. The *Bacillus subtilis* chromosome contains several loci that resemble origins (45). More than one of these loci (called DnaA box regions) functioning in *cis* may possibly be required for replication (44). Origins have been cloned and studied by origin search techniques which generate minichromosomes within homologous or heterologous hosts (48, 71, 76). In studies of members of the family *Enterobacteriaceae*,

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gene libraries were inserted into ColE1-type plasmids and then used to transform *E. coli polA* hosts unable to support replication of ColE1 replicons (23, 35, 61, 76). Under these conditions, the recombinants selected must have been chimeric, i.e., they contained another origin that could function when ColE1 could not (35).

Cloning of *C. burnetii* genes has been done exclusively in *E. coli* by complementation or by antibody screening (30, 63). Information has thus been gained regarding the organism's antigens (26, 33, 63), gene structure (24–26, 32, 43, 63), biotypes (40), stress proteins (63), and repetitive insertion sequences (31). Since transformation methods for *C. burnetii* were unavailable, origin search techniques with *E. coli* as a heterologous host were used, in much the same manner as was done with members of the family *Enterobacteriaceae* (23, 61, 76). Fragments generated by partial digestion of *C. burnetii* chromosomal DNA were ligated to a Kan^r marker and used to transform *E. coli*; transformation of the host to kanamycin resistance thus required that a *C. burnetii* element with origin function be ligated to the marker.

Origin search by this approach led to the isolation of an autonomous replication sequence (ARS) from *C. burnetii*. To verify that this ARS functioned as an origin, replication intermediates were sought (4, 73). Where initiation starts internally and is characterized by a theta form or "bubble eye" intermediate, identification and mapping is most often done by two-dimensional (2D) electrophoresis (4, 73). The ARS fragments isolated from *C. burnetii* grown in fibroblast cultures were run in 2D gels, transferred by Southern analysis techniques, and probed. Linear duplexes and Y forms were detected; however, no evidence for bubble eyes was found.

Deletion studies and sequencing revealed that the minimal ARS region contained DnaA boxes and A+T-rich direct repeats, plus other structures sometimes found in origins. However, it did not easily fit those general structural characteristics held in common by several bacterial chromosomal origins with respect to numbering and spacing of DnaA boxes (73, 74). The region surrounding the ARS possessed many open reading frames (ORFs), some of which demonstrated identity and order canonical to genes found in bacterial origin regions.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. Plasmid pBR322 and pBluescript vectors were obtained from GIBCO-Bethesda Research Laboratories (BRL) (Gaithersburg, Md.) and Stratagene, Inc. (La Jolla, Calif.), respectively.

L broth (38), SOB medium (21), or SOC medium (21) was used for cultivation of *E. coli*. The addition of 50 μg of kanamycin (Calbiochem Corp., San Diego, Calif.) per ml or 100 μg of ampicillin (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) per ml to the growth medium permitted selection of the antibiotic-resistant bacteria. The *E. coli* DH5αF′ provides for α complementation of β-galactosidase with pBluescript vectors, therefore permitting blue or white (Lac$^+$/Lac$^-$) color selection of plasmids containing inserts. SOC medium containing 100 μg of ampicillin per ml, 80 μg of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) per ml, and 20 mM IPTG (isopropyl-β-D-thiogalactopyranoside; GIBCO-BRL) was used in the selection of bacteria containing inserts.

Persistently infected BHK-21 cell lines were established and cultivated with *C. burnetii* Nine Mile strain phase I clone 7 (CB9MIC7) (68) as previously described (75). BHK-21 fibroblasts (ATCC CCL10) were obtained from the American Type Culture Collection.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype[a] and phenotype[b]	Source and/or reference
E. coli		
JZ279	F$^-$ *recA56 hsdR lacY galK2 galT22 metB1 trpR55 supE44 supF58*	J. Zyskind (23)
JZ294	F$^-$ *polA1 argH hsdR rpsL thyA36*	J. Zyskind (23)
DH5αF′	F′ *endA1 hsdR17* (r_K^- m_K^-) *supE44 thi-1 recA1 gyrA relA1* (*lacZYA-argF*) U169φ80d *lacZ* M15	Gibco-BRL
Plasmids		
pML21	*ori* (ColE1) Kan^r	J. Zyskind (28)
pJZ101	*E. coli oriC*, *ori* (ColE1) Amp^r	J. Zyskind (56)
pEMBL8(+)	*ori* (ColE1) *ori* (phage F1) *lac*$^+$ Amp^r	L. Dente (10)
pQpH201	Plasmid QpH1 from CB9MIC7 in the pHC79 cosmid vector	M. Vodkin
pSYC1	*C. burnetii* ARS, Kan^r	This study
pSYC2	*C. burnetii* ARS, Kan^r	This study
pSYC3	*C. burnetii* ARS, Kan^r	This study
pSYC101	*C. burnetii* ARS cloned into pEMBL8(+)	This study
pHTPAB	*C. burnetii htpAB* gene in pBluescript KS	T. Hoover

[a] Abbreviations used are those from Bachmann (2).
[b] Abbreviations for antibiotic resistance: Amp^r, ampicillin; Kan^r, kanamycin.

Enzymes and chemicals. *Eco*RI, *Not*I, *Sac*I, *Sac*II, and *Spe*I were purchased from New England Biolabs, Inc. (Beverly, Mass.). *Bst*XI was purchased from Promega Corp. (Madison, Wis.). All other restriction enzymes were purchased from GIBCO-BRL. All restriction endonuclease reactions were carried out as recommended by the suppliers. T4 DNA ligase (New England Biolabs, Inc.) and alkaline phosphatase (Boehringer Mannheim Biochemicals) reactions were carried out under conditions recommended by the suppliers. All other enzymes and kits used in the subsequent studies are described elsewhere in Materials and Methods. [α-^{32}P]dATP (5,000 or 800 Ci/mmol) was purchased from New England Nuclear (Wilmington, Del.), and [α-^{32}P]dCTP (3,000 Ci/mmol) was obtained from Amersham, Inc. (Arlington Heights, Ill.).

Isolation of chromosomal and plasmid DNAs. *C. burnetii* chromosomal DNA was extracted by a thermolysin-sodium dodecyl sulfate (SDS) procedure (52). Plasmid minipreps were prepared by an alkaline lysis procedure (41). A modified protocol for the pZ523 spin columns (5 Prime → 3 Prime Co., Boulder, Colo.) was used in the large-scale plasmid preparation (14). Extraction and preparation of DNA for analysis of replicative intermediates is described under "2D agarose gel electrophoresis."

DNA manipulations. To clone the *C. burnetii* ARS, *C. burnetii* chromosomal DNA was partially *Eco*RI digested and ligated, by using T4 ligase, to a kanamycin resistance fragment from plasmid pML21 (39). Plasmid pML21 digested with *Eco*RI produces two fragments, a larger 6.8-kb kanamycin resistance fragment and a smaller 3.3-kb ColE1 origin fragment. These were electrophoretically separated in 5% polyacrylamide, and the larger Kan^r fragment was excised, purified by electroelution in 1/20× TBE buffer (41), and used in the

ligation reaction. The *C. burnetii* ARS was subcloned into pEMBL8+ and pBluescript vectors and used in subsequent studies.

E. coli JZ279 and JZ294 were transformed by a calcium chloride protocol previously described (37). *E. coli* DH5αF′ was transformed by a standard high-efficiency protocol (21).

General electrophoresis, Southern transfers, and hybridizations. One-dimensional electrophoresis was performed as previously described (41), using 0.7 to 1.2% agarose gels or 5% polyacrylamide gels in TBE buffer. DNA fragments used for probe synthesis were electrophoresed and purified by electroelution (41).

Nylon membranes (Hybond-N; Amersham, Inc.) containing transferred DNA were prepared as specified. All membranes were baked at 81°C under a vacuum for 1.5 h. Hybridizations and washes were performed at 56°C for low-stringency conditions and at 68°C for high-stringency conditions (58). Nitrocellulose filters with transferred DNA were prehybridized and hybridized in formamide (50 and 45%, respectively), 5% dextran sulfate, and 100 to 200 μg of biotinylated probe (GIBCO-BRL) per ml at 42°C as recommended by the probe manufacturer. The nitrocellulose filters were washed in 0.16× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS under either low (56°C)-stringency or high (68°C)-stringency conditions. The immunological detection was performed as described by the manufacturer.

Probe synthesis. Biotinylated probes were synthesized by using a nick translation kit (the BluGene system) with biotin-11-dUTP (GIBCO-BRL) as described by the supplier and recovered by ethanol precipitation. Probes were also radiolabeled with [α-^{32}P]dCTP by a nick translation kit (51) (Boehringer Mannheim Biochemicals) or using a multiprimer DNA labeling system (Amersham, Inc.) (12, 13).

The PCR probes were generated as previously described (54). Ampli*Taq* polymerase and buffer was purchased from Perkin-Elmer Cetus (Norwalk, Conn.). The unincorporated nucleotides were removed by using Sephadex G-50 spin columns (41). The reaction mixture was analyzed by agarose gel electrophoresis, yielding a primary PCR product of the expected size.

Primers for PCR were synthesized by the West Virginia University Recombinant DNA Core Facility. The probe to the minimal ARS utilized the primers 5′-CCGAGGTGCGATTA AACGTG-3′ and 5′-CGATTCAGTACTTGACGGCC-3′ located at positions 2656 to 2675 and 3183 to 3202, respectively (7) (see Fig. 3). The probe outside of the minimal ARS, located in the region showing nucleotide sequence homology to *glyS*, was generated with the primers 5′-GCAGGAGA TCAATTACTCG-3′ and 5′-TCGGCATCGGAATCAAAGA G-3′ at positions 105 through 124 and 606 through 625, respectively. The probe to the heat shock protein was synthesized with the primers 5′-CAACCGGCCATGTTATGACG-3′ and 5′-GCGTGTAATACCTCGTGGGA-3′ located at positions −131 to −112 and 343 to 362 from the previously published sequence (63). Primers to *E. coli oriC* included 5′-GGTGATTGCCTCGCATAACG-3′ and 5′-TCCGGTGC AAAACAGACAGG-3′ located at positions −88 to −68 and 380 to 401, respectively, from the previously published sequence (42).

Unidirectional deletion of the *C. burnetii* ARS fragment. The *C. burnetii* 5.8-kb fragment was subcloned into pBluescript vectors and unidirectionally deleted by exonuclease III and nuclease S1 treatment (50) using the Erase-A-Base system as directed by the supplier (Promega). DNAs containing deletions were purified by electrophoresis in 1.0% low-melting-temperature agarose with extraction of the band and subsequent religation by using T4 ligase as directed by the supplier. The DNAs were transformed into *E. coli* DH5αF′ and subjected to a quick screening as recommended. Colonies were grown overnight, and each was resuspended in 20 μl of STE buffer (100 mM NaCl, 10 mM EDTA, 20 mM Tris-HCl [pH 7.0]), extracted with an equal volume of phenol-chloroform (1:1, vol/vol), treated with RNase A, and analyzed by agarose gel electrophoresis to determine the size of the deletion. ARS function was determined by transformation of *E. coli* JZ294 (*polA*). The unidirectionally deleted plasmids were then sequenced to determine the exact deletion.

Nucleotide sequence determination. Plasmids carrying the various exonuclease III-S1-deleted fragments were sequenced (27, 53). The double-stranded DNA templates were prepared by a modified small-scale alkaline lysis procedure (36). Primer T3 (or reverse primer) and primer T7 (or M13 primer) were used for sequencing the two DNA strands; the reannealing reactions were carried out at 37°C for 30 min. Sequenase (United States Biochemical Corp. [60]) was used for dideoxynucleotide chain termination by the method of Sanger et al. (53) as specified by the supplier. DNA sequence analysis and amino acid sequences of potential ORFs were analyzed with Ig Suite, IntelliGenetics and UWGCG packages (11) or with NUSCAN and PROSCAN through DNAstar using the GenBank database. The DNA sequence and the ORF amino acid sequences were aligned by the Wilbur-Lipman, AALIGN (Lipman-Pearson FASTP algorithm), and Needleman-Wunsch methods (47, 67).

2D agarose gel electrophoresis. Intracellular and extracellular *C. burnetii* organisms were obtained as previously described (75). *E. coli* was grown in L broth until an optical density of approximately 0.5 at 620 nm was obtained (an exponentially growing culture, 2×10^8 cells per ml) and was treated with sodium azide at a final concentration of 0.1%.

The chromosomal DNA of *C. burnetii* was isolated by an alkaline phenol extraction (64). Chromosomal DNA from *E. coli* was isolated by modification of a protocol described for *Saccharomyces cerevisiae* (29). Cells were resuspended (approximately 1 mg/ml) in lysozyme (1 mg/ml) plus 50 mM glucose–25 mM Tris-HCl [pH 8.0]–1 mM EDTA and incubated for 30 min at 37°C. Cells were pelleted, slowly resuspended in 4.5 M guanidine hydrochloride–0.1 M EDTA–0.15 M NaCl–0.05% Sarkosyl, pH 8.0, and incubated at 60°C for 20 min. The nucleic acids were precipitated with ethanol, resuspended, and treated with RNase A and proteinase K before the DNA was precipitated as previously described (29).

DNA was digested with restriction enzymes as recommended by the manufacturers. The 2D gels were run by a modification of a previously described procedure (4). For detection of fragments larger than 7.0 kb, digested DNAs were electrophoresed with 0.35% agarose in the first dimension. The lane was excised and polymerized with 0.8% agarose for the second dimension. For the detection of fragments of between 4 and 7 kb, digested DNAs were electrophoresed with 0.4% agarose for the first dimension and 1.0% agarose for the second dimension. The first dimension was electrophoresed for approximately 15 h at room temperature at 0.37 V per linear cm with molecular weight markers. The second dimension was run at 2.3 V per linear cm for 10 to 15 h under refrigeration at 4°C. The DNA was transferred to Hybond-N nylon membranes (Amersham, Inc.) with 20× SSC. After baking, the membranes were prehybridized for 5 to 8 h at 68°C in hybridization solution (5× SSC, 0.1% *N*-lauroylsarcosine, 0.02% SDS, and 0.7% blocking reagent [Boehringer Mannheim]). Hybridization was carried out in the same solution containing 6×10^6 to 1×10^7 cpm of PCR-generated or nick-translated probe for 18

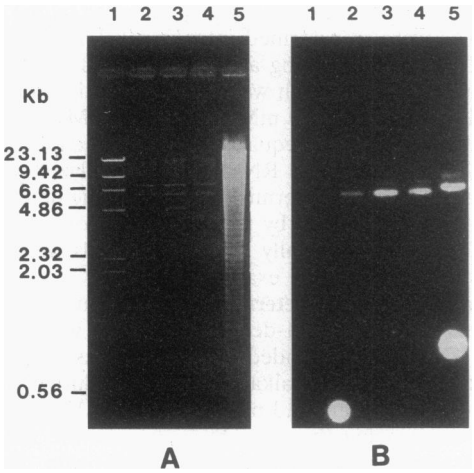

FIG. 1. (A) Ethidium bromide staining of DNAs. (B) Southern blot hybridization using *C. burnetii* ARS as probe. The specific radioactivity of the probe was 6.5×10^8 cpm/μg. During hybridization, 10^6 cpm of the probe was used, and autoradiographic exposure was for 18 h. Lanes 1, *Hin*dIII digest of lambda DNA; lanes 2, *Eco*RI digest of pSYC1; lanes 3, *Eco*RI digest of pSYC2; lanes 4, *Eco*RI digest of pSYC3; lanes 5, *Eco*RI digest of *C. burnetii* chromosomal DNA.

to 22 h at 68°C. The blots were washed twice for 10 min in 2× SSC–0.1% SDS at room temperature and twice for 15 min in 0.1× SSC–0.1% SDS at 68°C. Exposure to Kodak XAR-5 film was performed for 11 to 14 days at −80°C.

Nucleotide sequence accession number. The GenBank accession number for the *C. burnetii* 5.8-kb ARS sequence is U10529.

RESULTS

Cloning of the *C. burnetii* ARS. The ARS from the *C. burnetii* chromosome was cloned by selecting a DNA fragment capable of initiating DNA replication autonomously in the origin search plasmid, pML21 (39). The Kan^r fragment, derived from *Eco*RI digestion of pML21, was ligated to an *Eco*RI-generated partial digest of *C. burnetii* chromosomal DNA. *E. coli* JZ279 was transformed to kanamycin resistance. Three recombinants, pSYC1, pSYC2, and pSYC3, all contained a 5.8-kb fragment (Fig. 1A, lanes 2, 3, and 4, respectively). Hybridization using the 5.8-kb fragment derived from pSYC1 as a probe indicated that this DNA fragment appeared in all three plasmids and in the *C. burnetii* chromosome (Fig. 1).

To eliminate other vectors and bacteria used in the study as possible source contaminants responsible for ARS activity and to further ascertain the source and the uniqueness of the ARS, a series of hybridization experiments was done. These experiments were designed to search for primary structures homologous to the cloned ARS in (i) the *C. burnetii* Nine Mile strain's resident plasmid QpH1 (plasmid pQpH201 contains QpH1 in cosmid pHC79), (ii) the origin search plasmid pML21, (iii) the *E. coli oriC*-containing plasmid pJZ101, (iv) the ColE1-type replicon in the *E. coli* plasmid pBR322, and in the genomic DNA of selected species of other gram-negative bacteria, including (v) *Salmonella typhimurium*, (vi) *Klebsiella pneumoniae*, (vii) *Enterobacter aerogenes*, and (viii) *E. coli* (Table 2). Except in the positive controls, no detectable hybridization signals were found in any of these DNAs (Table 2); on the other hand, use of a probe composed of *E. coli oriC* did show a hybridization signal to *Eco*RI-generated chromosome fragments from *C. burnetii* (data not shown). The results suggested that the *C. burnetii* ARS was unique and derived only from the organism's chromosome.

TABLE 2. Hybridization analysis

DNA source	Hybridization result[a]				
	C. burnetii ARS		*E. coli oriC*		pBR322 ori,[b] high
	Low	High	Low	High	
pQpH201	ND	−	ND	ND	ND
pBR322	ND	−	ND	ND	+
pML21	ND	−	ND	ND	+
pJZ101	ND	−	ND	ND	ND
pSYC1	ND	+	−[c]	−	−
C. burnetii	+	+	−[c]	−	−
E. coli	−	ND	+	+	−
S. typhimurium	−	ND	+	−	ND
K. pneumoniae	−	ND	+	−	ND
E. aerogenes	−	ND	+	−	ND

[a] Results are indicated for the probes and stringency conditions (high or low) used. Abbreviations: ND, not done; +, hybridization clearly detected; −, no hybridization detected.
[b] The *Sau*3AI-*Nci*I fragment from pBR322 containing the ColE1 origin (ori) from pBR322 was used as a probe.
[c] Faint hybridization was detected.

Minimal ARS region determination. The 5.8-kb *C. burnetii* *Eco*RI fragment containing the ARS was inserted into the *Eco*RI site of the pBluescript plasmids (KS$^+$, KS$^-$, SK$^+$, and SK$^-$; Stratagene). The derivative plasmids were digested with the appropriate restriction enzymes and subjected to unidirectional exonuclease III digestion and S1 nuclease treatment, followed by religation and transformation into DH5αF′ to generate progressively larger deletions from either end. Certain constructs were subsequently subjected to the same procedure at the opposite end of the insert to generate deletions at both ends. The resulting plasmids with various portions of either or both ends deleted were used in DNA sequence analysis and to determine the minimal region required for autonomous replication. These plasmids are summarized in Fig. 2A.

The minimal ARS region was determined by replication of the pBluescript clones carrying various segments of the 5.8-kb *C. burnetii* fragment in *E. coli* JZ294, a *polA* strain that does not support replication from ColE1 origins. Thus, ampicillin resistance depends upon a functional alternate origin within the inserted DNA, and colonies that grew on LB plates supplemented with ampicillin were deemed to have sufficient ARS DNA to allow replication of the plasmid. The region required for replication was narrowed by examination of clones with deletions in either end of the insert (Fig. 2B) and further narrowed by analysis of smaller incremental deletion derivatives of pKOR1, a 568-bp restriction fragment derived from deletion plasmid pKSO(−)1311a. It was thus deduced that the minimal region required for ARS activity extended from between positions 2726 and 2769 to a point between positions 3083 and 3128.

Sequence of the ARS region. The entire *Eco*RI 5.8-kb fragment was sequenced and numbered (1 to 5809) by the use of plasmids carrying the progressively deleted DNA fragments. The nucleotide sequences determined in each strand of the minimal region are shown within the brackets in Fig. 3. The strategy (data not shown) included the sequencing of each strand; some overlapping segments were sequenced three or four times. Several structures characteristic of a bacterial

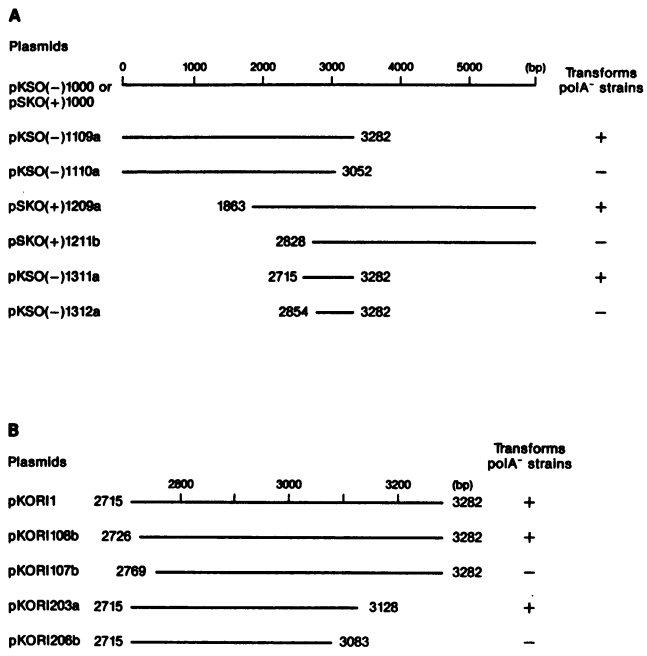

FIG. 2. The exonuclease III-S1 deletion products. The horizontal line indicates the *C. burnetii* DNAs carried by each plasmid. The ability to transform *E. coli* JZ294 is shown, indicating successful transformation (+) or inability to transform (−). The initial deletion fragments are demonstrated (A), and the subsequent deletions to define the boundaries of the minimal ARS are shown (B). Note that the scales are different in panels A and B.

chromosomal origin were found within this sequence. The presence of DnaA boxes for the binding of *dnaA* gene protein product were expected, and two that possessed complete consensus sequences, i.e., TTATCCACA, were found (16) (Fig. 3); the two DnaA boxes are located at nucleotides 2745 through 2737 (leftwards) and 2784 through 2792 (rightwards). At least two and possibly three A+T-rich direct repeats were found. They were 21-mers and similar to those utilized in other origins for directing DNA helicase into an initiating replication fork. These are located to the right of both DnaA boxes, at nucleotides 2824 through 2844, 2857 through 2877, and 2916 through 2936 (Fig. 3). GATC sequences, employed for methylation by *dam* methylase in enteric bacterial chromosomes (56), were not present: instead, six TTAA sequences were found between nucleotides 2889 and 2934. No ORFs were found within or spanning the minimal ARS region, even though there were two potential promoters located at the left and right ends of the structure. These were designated promoter left (P_L) and promoter right (P_R), respectively, and are directed outwards from the ARS. P_R is the probable promoter for the *rpmH* gene (as discussed below) and is located within the minimal sequence. A consensus nucleotide sequence for IHF (9) binding was located at positions 3077 through 3089. Another consensus sequence for FIS (34) was found at nucleotides 2984 through 2999.

Additional sequences were found to be similar to portions of the vegetative origins in the miniF and R6K plasmids. These are located at nucleotides 2553 through 2575, where a region nearly identical to a portion of the *oriS* in miniF was found, and at nucleotides 2836 through 2846 and 2871 through 2880, where shorter sequences similar to miniF and R6K origins, respectively, were revealed.

The overall A+T content of the minimal ARS is 63.3%. This is much higher than that seen in the *E. coli oriC*.

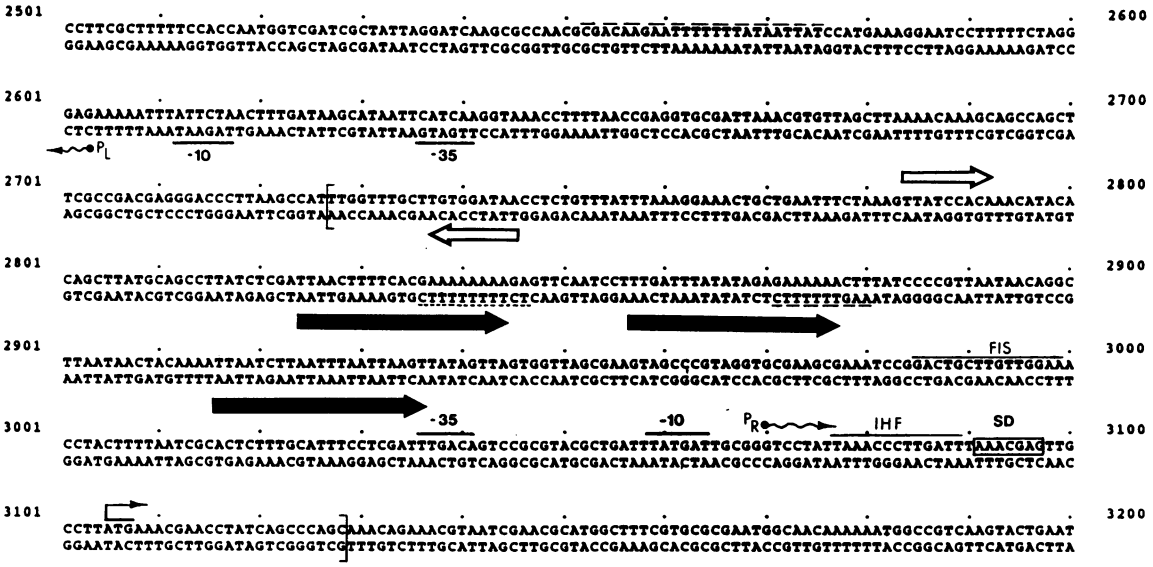

FIG. 3. Nucleotide sequence of the region required for autonomous replication of the *C. burnetii* chromosome. Nucleotide numbers are the same as those described in the text. The brackets indicate the minimal ARS region. The two DnaA boxes are indicated by open arrows. The 21-bp A+T-rich repeats are designated with thick solid arrows below the sequences. The two theoretical promoters (P_L and P_R) near the minimal ARS region and their −35 and −10 regions (Pribnow boxes) are shown. The boxed region indicates the potential Shine-Dalgarno (SD) sequence. Potential consensus sequences for FIS (34) and IHF binding sites (9) are indicated by thin lines above the consensus sequence and labeled accordingly. The sequences showing similarity to F1 miniplasmid are located at positions 2552 through 2575 and 2871 to 2880; these are designated with dashed lines located above and below the sequences, respectively. A short sequence showing similarity to a sequence within R6K is located at positions 2836 through 2846 and is designated with a dotted line below the sequence.

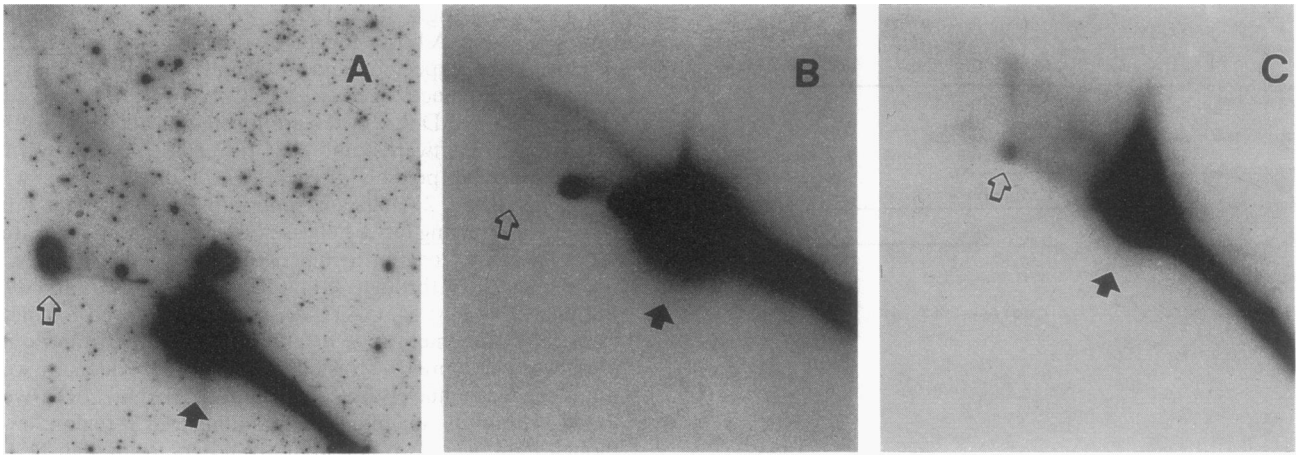

FIG. 4. 2D gel analysis of *E. coli oriC* and the *C. burnetii* ARS. Linear duplex DNA (solid arrow) and the point two times the size of the unreplicated fragment (open arrow) are indicated. (A) Two micrograms of *E. coli* DNA was restricted with *Eco*RI and analyzed by the 2D gel technique. A 546-bp fragment containing *oriC* was generated by PCR, electrophoresed, purified by electroelution, and used in a nick translation reaction resulting in a specific activity of 3.0×10^8 cpm/μg of DNA. Probe containing 1.7×10^7 cpm was used in the hybridization. The exposure time was 14 days. (B) Results of an *Eco*RI digest generating a 5.8-kb fragment containing the *C. burnetii* ARS nearly symmetrically located, probed with both the 547-bp minimal ARS and the 619-bp probe to the right of the ARS are shown. The exposure time was 11 days. The location of the 5.8-kb ARS fragment (solid arrow) and the return of the Y arc to the linear duplex DNA diagonal (open arrow) are indicated. The spot to the right of the open arrow in panel B results from incomplete digestion. (C) Results of a *Bam*HI-*Cla*I double digest generating a 5.0-kb fragment containing the *htpAB* genes hybridized with a 464-bp probe to the *htpA* gene are shown. The exposure time was 11 days. In panels B and C, 2D gels contained 1.5 μg of chromosomal DNA from a mixture of NRCb and MRCb cells; they were probed with 6×10^6 cpm of the appropriate PCR-generated probe.

Function of the ARS in the *C. burnetii* chromosome. Cell division cycle synchrony within this parasite during growth in hosts was not attainable. In addition, the time required for initiation of DNA synthesis and subsequent chromosome completion within a division cycle was expected to be relatively short, assuming that polymerase efficiency and speed in *C. burnetii* resembled those in conventional bacteria. These characteristics excluded the use of convenient approaches to study origin function. But one available method that does not require synchrony or a large amount of DNA is neutral-neutral 2D gel electrophoresis to detect replicative DNA forms. *C. burnetii* were grown in fibroblast cultures under growth enhancement conditions (high CO_2) as described previously, and two populations were harvested (75). DNA was extracted from the extracellular and intracellular populations by an alkaline phenol technique, restricted, and electrophoresed by methods previously used to visualize yeast replicative intermediates (4).

In order to establish a reference pattern for a bubble arc, a restriction fragment population containing a random mixture of replicating and nonreplicating *E. coli oriC* was resolved by electrophoresis, transferred, and probed (Fig. 4A). The prominent spot on the film marks the location of the 9.4-kb *Eco*RI linear duplex (nonreplicating) fragment (solid arrow). An arc representing a growing bubble or replication eye form (also see the diagram in Fig. 5C) is seen to originate at or near this spot and to trail upwards and to the left; the migration of the growing bubble was thus retained proportionately to its mass in the first dimension and proportionately to both shape and mass in the second dimension. This arc signal was not observed to return to the linear duplex arc at a point twofold greater than its original mass (Fig. 4A), a feature that would have been expected for a simple growing Y form (Fig. 5B). *C. burnetii* chromosomal DNA extracted from naturally released (NRCb) cells and mechanically released (MRCb) (75) cells was digested with *Eco*RI, electrophoresed in two dimensions, transferred to nylon membranes, and hybridized with a PCR-generated probe located internal to the 5.8-kb ARS fragment (Fig. 4B). Unlike the bubble arc observed for *E. coli oriC* shown in Fig. 4A, the signal observed was a shallow arc

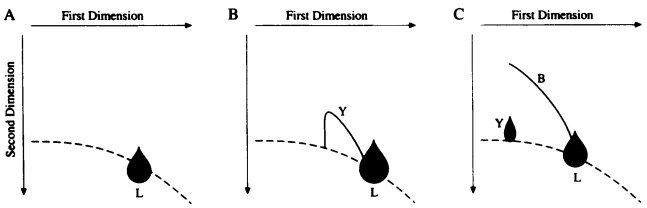

FIG. 5. Schematic diagrams of the patterns of replicative intermediates obtained by neutral-neutral 2D gel electrophoresis. The autoradiographic patterns after probe hybridization are shown in each panel, demonstrating patterns for restriction enzyme-digested DNA. The linear duplex DNA fragment of interest is labeled with an L and replicative intermediates are designated Y or B, indicative of either Y structures or bubble forms, respectively. The linear duplex DNA visualized by ethidium bromide staining is depicted as a dashed line. (A) When replicative intermediates are not detected, the linear DNA forms of the restriction fragment demonstrate a strong hybridization with the probe and no arc patterns are revealed. (B) A complete Y arc is generated by a replication fork, growing from an origin outside of the fragment, traversing the fragment. The Y arc rises up from the linear duplex DNA in a leftward manner, peaking with replicative intermediates containing branches of equal size. Intermediates where replication has proceeded further become more like linear forms and return to the linear duplex DNA signal. (C) A bubble arc is obtained when a restriction fragment serves as the origin of replication. Bubble intermediates rise from the linear duplex DNA in a manner such that the bubble arc is located above the region of the Y arc. If the origin is asymmetrically located with respect to the restriction sites and bidirectional replication is occurring, molecules that have undergone replication beyond one of the restriction sites will yield Y structures; these molecules form a partial Y arc discontinuous with the bubble arc.

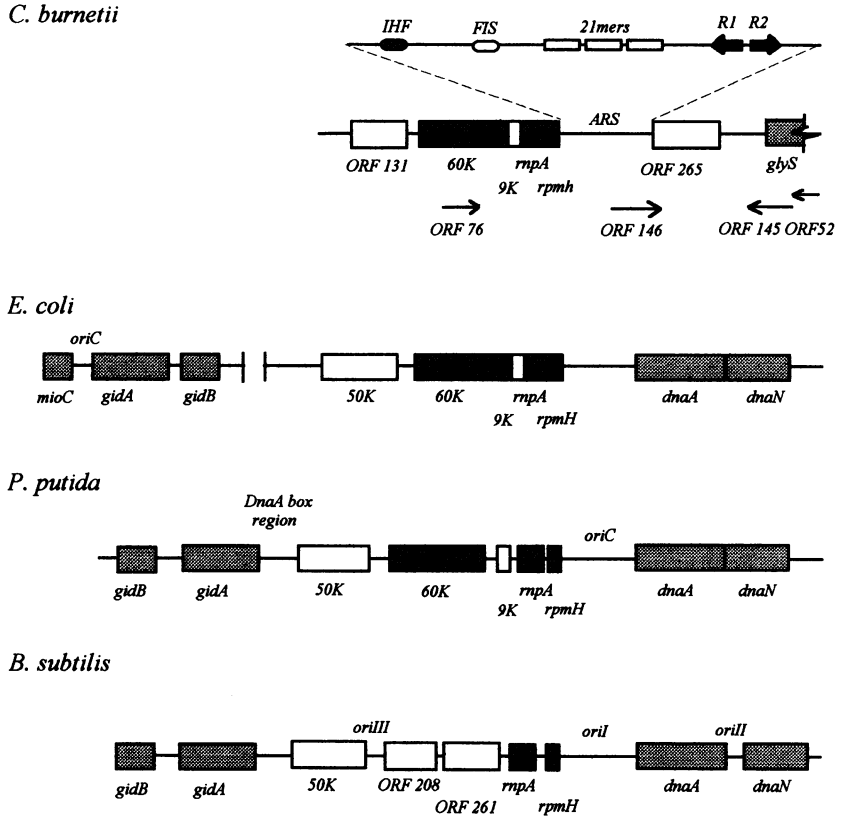

FIG. 6. The gene organization surrounding the *C. burnetii* ARS in comparison with the origin regions of *E. coli*, *P. putida*, and *B. subtilis*. Transcription from all *C. burnetii* ORFs is directed leftwards unless otherwise indicated by an arrow. The direction of transcription is directed away from the *P. putida oriC*, from *B. subtilis oriI*, and from the region analogous to origins in *E. coli*. The ORFs showing identity to established bacterial genes are labeled and/or designated by shaded boxes. The darker shaded boxes for the 60K, 9K, *rnpA*, and *rpmH* regions are provided for comparison. The ORFs with no significant homology to other genes are indicated by open boxes or arrows. The partial *glyS* gene shown for *C. burnetii* is designated as such on the basis of nucleotide sequence similarity to *E. coli glyS*. The *C. burnetii* minimal ARS is shown enlarged above the ARS region. The following possible origin motifs located in the ARS are indicated: DnaA boxes (solid arrows), 21-bp AT-rich repeats (open boxes), the FIS consensus sequence (open oval), and the IHF consensus sequence (shaded oval). The percent amino acid identity of the *C. burnetii* ORFs showing significant homology to other bacterial genes or ORFs is as follows. *C. burnetii rpmH* (44 amino acids [aa]) shows 84 and 80% identity with *E. coli rpmH* (46 aa) and *P. putida rpmH* (44 aa), respectively. *C. burnetii rnpA* (121 aa) shows 39 and 34% identity with *E. coli rnpA* (119 aa) and *P. putida rnpA* (133 aa) in 109- and 122-aa overlaps, respectively. The *C. burnetii* 9K protein (88 aa) demonstrates 62, 53, and 51% identity with *E. coli* (85 aa), *P. mirabilis* (86 aa), and *P. putida* (81 aa) 9K in 66-, 86-, and 81-aa overlaps, respectively. The *C. burnetii* 60K protein (487 aa) demonstrates 40% identity with both *E. coli* (548 aa) and *P. putida* (560 aa) 60K in a 495-aa overlap in each. The homology is made on the basis of the amino acid sequences deduced from the nucleotide sequences (15, 22, 42, 55, 73).

originating in the 5.8-kb linear duplex spot (solid arrow) and returning with a faint signal to the linear duplex diagnal at a point corresponding to 11.6 kb (open arrow, Fig. 4B). This signal is typical of a growing Y arc. Another control, this one to ascertain the pattern generated by the *C. burnetii* chromosomal region containing the *htpAB* operon, was performed; a DNA fragment excised from this region would be expected to exist primarily as a linear duplex and occasionally as a growing Y when a replication fork moved through it. The experiment was performed with DNA extracted from NRCb and MRCb and restricted with *Bam*HI and *Cla*I to generate a 5.0-kb piece. After electrophoresis, transfer, and hybridization with a PCR-generated probe from the *htpAB* (heat shock) promoter region, a pattern (Fig. 4C) that was very similar to (and superimposable upon) the ARS pattern (Fig. 4B) was generated. This DNA pattern obtained from the heat shock operon's promoter region did show a more defined return signal (Fig. 4C); however, it was not otherwise remarkably different from that obtained from the ARS.

These results, which were unexpected, are consistent with the interpretation that the *C. burnetii* ARS rescued by origin search techniques in *E. coli* is not an origin in *C. burnetii*.

Structure of the chromosomal neighborhood surrounding the *C. burnetii* ARS. Previous work (7) had located nine potential reading frames, each with Shine-Dalgarno-like sequences, within the 5.8-kb ARS fragment; seven were found reading to the right and two to the left. The most interesting feature discovered in that early analysis was the nucleotide similarity of the 3'-terminal 863 nucleotides of the 5.8-kb ARS fragment to nucleotide positions 2813 to 3171 of the *E. coli glyS* gene (Fig. 6) (7). In the *E. coli* chromosome, *glyS* is located at 80 min and *oriC* is at 84 min (2). More recent assignments using DNAstar to access GenBank and to run alignment and sequence comparisons revealed probable genetic loci within the *C. burnetii* ARS fragment (Fig. 6). As can be seen, identities can now be assigned to four ORFs within the *C. burnetii Eco*RI-generated ARS fragment; the significance, if any, of the unassigned ORFs remains obscure. Most notable

are the locations of the *rpmH*, *rnpA*, 9K, and 60K gene functions; all of these have been located directly adjacent to the chromosomal origin in *Pseudomonas putida* (57, 73) and are located there in the same order and transcriptional direction (away from the ARS or origin) as found here (Fig. 6). In *E. coli*, *rpmH* is the gene encoding the 50S ribosomal subunit L34 and *rnpA* is ribonuclease P, while the 9K gene function is unknown; the 60K locus encodes an inner membrane protein (22). In *E. coli* where the arrangement of these genes (in order and direction of transcription) is *rpmH-rnpA*-9K-60K, the cluster is found next to a single perfect DnaA box that is located over 40-kbp away from *oriC* (Fig. 6). This same gene cluster is located immediately adjacent to a promoter for *dnaA*, rather than an ARS, in *E. coli*.

Although the structure surrounding the *C. burnetii* ARS is reminiscent of a chromosomal origin, functional evidence for an origin was not obtained.

DISCUSSION

An autonomous replication sequence has been isolated from *C. burnetii* by an origin search, plasmid rescue technique. Three independent clones, all able to replicate kanamycin resistance in *E. coli* and all containing the same 5.8-kb piece of *C. burnetii* chromosomal DNA, were obtained. By various transfer and hybridization experiments, the cloned DNA was shown to lack significant homology to origins from other gram-negative bacteria. The ARS functioned in an *E. coli polA* host, which aided experiments in determining its approximate smallest effective size. Complete sequencing of both strands by an overlapping strategy revealed both structural similarities and dissimilarities to other origins and their regions.

These cloning and sequence data established the following characteristics concerning the *C. burnetii* ARS. (i) It is found in a 5.8-kb *Eco*RI fragment on the *C. burnetii* chromosome, (ii) it is not a ColE1 type of replicon origin, (iii) it is not found in the organism's resident plasmid QpH1, and (iv) it lacks significant homology to chromosomal origins in gram-negative bacteria.

Nucleotide sequence analysis and database searches have revealed the likely existence of a 60-kDa inner membrane protein gene and a portion of a glycine tRNA ligase (*glyS*) gene neighboring the minimal ARS (Fig. 6). ORFs with sequence structure similarity to the *rnpA* and the *rpmH* genes have also been revealed. By comparison with chromosomal origins in other bacteria, the probable existence of these genes in locations near to the ARS provides circumstantial evidence that the ARS found within this region is a chromosomal origin (Fig. 6). The gene order *rpmH-rnpA*-9K-60K, with *rpmH* located directly adjacent to *oriC*, and with transcription of all four directed away from *oriC*, comprises a motif seen in *Pseudomonas* species (Fig. 6, *P. putida*). In *E. coli*, this organization is duplicated, except that *oriC* is replaced by a sequence containing only one or a few DnaA boxes within a promoter region for the *dnaA* gene. Except for a lack of the *dnaA* gene, this same organization is apparent in the *C. burnetii* ARS region (Fig. 6). In *B. subtilis*, only part of this motif, *rpmH-rnpA*, is located adjacent to one of the origins, *oriI*. In most bacterial origin regions, the DnaA protein gene is located exactly adjacent to *oriC*, on the side opposite the *rpmH-rnpA*-9K-60K group. Sequence evidence for this location of *dnaA* in *C. burnetii* was not found; neither ORF 146 nor ORF 265 possessed significant homology to *dnaA* (Fig. 6).

The results obtained from blots of 2D gels wherein *C. burnetii* chromosomal DNA was electrophoresed do not demonstrate the presence of initiating theta forms in the ARS (Fig. 4B). The experiment was repeated several times, and arc patterns that would suggest the presence of either an asymmetric or symmetric bubble eye (small or young theta forms) have never been detected, regardless of the probe used. Instead, all of the patterns observed can be best explained as representative of a simple Y arc indicative of a fragment through which a single replication fork passes. To ascertain the validity of this conclusion, a single DNA preparation was probed by a PCR-derived sequence homologous to a region of the heat shock *htpAB* operon. The pattern obtained from this 2D agarose gel analysis was taken to represent one typical of a locus outside a replication origin in the *C. burnetii* chromosome, wherein an occasional replication fork generating a Y arc would be found. This pattern was very similar to that seen for the *C. burnetii* ARS chromosomal 2D gel results.

In addition to the lack of functional evidence for a chromosome replication origin, the sequence (primary) structure of the *C. burnetii* ARS likewise does not suggest chromosomal origin function. From the analysis of chromosomal origins described for the members of the family *Enterobacteriaceae* (76), *Pseudomonas* species (73), and *B. subtilis* (45), a set of fundamental features common to bacterial chromosomal origins emerges (57, 73); these features are (i) a minimum of four DnaA binding sites, (ii) two of which (R1 and R4) are separated by approximately 180 bp and are inverted repeats, (iii) two or more DnaA boxes present between R1 and R4, and (iv) three 13- to 16-bp AT-rich direct repeats found adjacent to the R1 site but not within the R1-R4 region. The ARS for *C. burnetii* fails to meet at least two of these criteria: there are only two DnaA sites, and although they are inverted repeats they are separated by much less distance, only 38 bp. The *C. burnetii* ARS does possess at least two of the three requisite AT-rich direct repeats, located to the left of, and not between, the DnaA boxes. One repeat in the A+T-rich region shows a 10-in-13-bp homology to one found in *Pseudomonas aeruginosa*.

Although the structural data for the chromosomal neighborhood surrounding the 5.8-kb region could be consistent with an origin assignment, the functional analysis and ARS sequence analysis within the 5.8-kb fragment provide direct and indirect evidence against origin function. The apparent incongruence between these three sets of data warrants some consideration of the appropriateness of the 2D gel techniques in the determination of function.

There was every reason to suspect, in the present work, that intracellular *C. burnetii* presented problems for the 2D technique. Unlike times for most conventional bacteria growing axenically, the generation time for *C. burnetii* growing intracellularly is more similar to that of cultured eucaryotic cells (59). The generation time of the organism in the present studies was about 18 h. Presuming that slowly growing bacteria initiate chromosomal replication infrequently but once initiated, elongate chromosomes at a consistently fast rate regardless of growth conditions, it would be expected that the population of chromosomes examined here might contain proportionately fewer (probably 100-fold less!) replicating chromosomes than were found in other studies utilizing this technique for conventional, axenically grown bacteria. Given that the technique additionally requires that a significant proportion of such replicating chromosomes be caught in the "window" of initiation and fork movement within the fragment examined, it becomes clear that the method may be marginal here: true replicative forms, after being probed as described, may be eclipsed by the proportionately massive signal given by fragments lacking replication activity. Examination of lighter exposures minimizing the linear duplex DNA signal revealed no evidence of bubble intermediates. Since Y-replicative inter-

mediates are detected, bubble arcs (if present) should be just as easy to detect because they should be affected even less by diffusive background noise from the linear duplex spot. The Y arcs obtained with the ARS and the *htpAB* gene are nearly identical in shape, size, and arc pitch. The evidence is consistent with the ARS not serving as the origin of DNA replication.

Isolation of an ARS which does not function as an origin has been observed previously. When origin search techniques were used for *P. putida* and *P. aeruginosa*, one ARS structure was obtained from the former and two from the latter. Subsequent testing by 2D agarose techniques revealed that only one ARS from *P. aeruginosa* functioned as a chromosomal origin. The significance of ARS structures that function for plasmid replication within a host but do not initiate chromosome replication is presently unknown. In the present case, it is tempting to speculate that the ARS functions in *C. burnetii* as an alternate chromosomal origin under other growth conditions, such as in natural ungulate or arthropod hosts. *C. burnetii* naturally infects both arthropod and mammalian hosts. Thus, use of separate origins under conditions of differing nutrition and temperature is a distinct possibility.

Alternatively, the possibility that the *C. burnetii* ARS represents a functional origin for another genetic element or replicon, such as an integrated plasmid or phage, was considered. In addition to the similarity to R6K and miniF plasmid, the *C. burnetii* ARS has sequences similar to the nick sites found in origins of single-stranded DNA plasmids from gram-positive (18) and gram-negative (70, 72) bacteria as well as bacteriophages from gram-negative bacteria. The similarity includes two *C. burnetii* sequences, GTTTGCTTGTGGATA ACCT and GTATGTTTGTGGATAACT, which overlap the DnaA boxes in the *C. burnetii* ARS. Analysis of the *E. coli oriC* and the *P. aeruginosa oriC* and ARS reveals that similar sequences are present in origins which do not initiate via a nicking mechanism. Thus, by analogy, the role of similar sites (if any) for replication of the *C. burnetii* ARS is uncertain. The probable existence of several chromosomal gene loci identified adjacent to the *C. burnetii* ARS region (Fig. 6) also argues against a role within an integrated plasmid or phage.

It was originally proposed (7) that the *C. burnetii* ARS was the chromosomal origin. In order to test this hypothesis, three avenues, comprising either indirect or direct evidence, were followed. It was expected that an ARS with origin function should show (i) existence of bubble arcs in 2D gel electrophoresis. In addition, supporting indirect evidence comprises (ii) spacing and the number of DnaA boxes in accordance with those seen to be in common with other bacterial origins and (iii) evidence, from sequence data of neighboring genes, that gene occurrence and order around the origin resemble those characterized in other bacteria. A fourth approach to study the origin hypothesis would be to investigate ARS's function as a possible minichromosome. Although determination of a replication mode by reintroduction of the ARS into *C. burnetii* was not possible, a study of the replication of pSYC1 in *E. coli* JZ279 could reveal interesting clues about its role. In preliminary work, 2D gel analysis of pSYC1 replication intermediates isolated from *E. coli* showed complicated, multiple arc patterns that were not easily interpreted. In that host at least, the mode by which the ARS directs replication in pSYC1 therefore remains uncertain; however, the mechanism does not appear to be simple. Thus, attempts to determine if pSYC1 functions as a minichromosome, i.e., initiates replication bidirectionally from an internal origin, remain inconclusive. Further studies on this and other aspects of the *C. burnetii* ARS are ongoing.

The data are inconsistent with a role for the ARS as a chromosomal origin. It is possible that this ARS, under some other conditions (e.g., in natural hosts), functions as an origin.

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