

## Physical and Genetic Map of the Obligate Intracellular Bacterium *Coxiella burnetii*

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**Pulsed-field gel electrophoresis and PCR techniques have been used to construct a *NotI* macrorestriction map of the obligate intracellular bacterium *Coxiella burnetii* Nine Mile. The size of the chromosome has been determined to be 2,103 kb comprising 29 *NotI* restriction fragments. The average resolution is 72.5 kb, or about 3.5% of the genome. Experimental data support the presence of a linear chromosome. Published genes were localized on the physical map by Southern hybridization. One gene, recognized as transposable element, was found to be present in at least nine sites evenly distributed over the whole chromosome. There is only one copy of a 16S rRNA gene. The putative *oriC* has been located on a 27.5-kb *NotI* fragment. Gene organization upstream the *oriC* is almost identical to that of *Pseudomonas putida* and *Bacillus subtilis*, whereas gene organization downstream the *oriC* seems to be unique among bacteria. The physical map will be helpful in investigations of the great heterogeneity in restriction fragment length polymorphism patterns of different isolates and the great variation in genome size. The genetic map will help to determine whether gene order in different isolates is conserved.**

*Coxiella burnetii*, an obligate intracellular bacterium propagating in the phagolysosomes of eucaryotic cells, is the only species of the genus *Coxiella*. In comparison to other members of the family *Rickettsiaceae*, *C. burnetii* demonstrates high resistance to chemical and physical agents, making it possible for the organism to remain infectious after years outside the host cell (2). *C. burnetii* infection of humans manifests itself as acute Q fever with influenza-like symptoms. However, infection may also result in the therapy-resistant chronic form of Q fever with endocarditis, granulomatous hepatitis, or osteomyelitis (37).

Though *C. burnetii* has been recognized worldwide as an important pathogen, little is known about the genes that contribute to virulence, particularly tissue invasiveness and intracellular persistence. Virulence potential is correlated with lipopolysaccharide (LPS) content of the organism (55). When propagated in nonimmunocompetent systems such as embryonated chicken eggs or persistently infected cell cultures, *C. burnetii* converts from phase I to phase II particles. Phase shift is accompanied by a drastic change in LPS content and structure of the outer membrane, with a significant decrease in virulence potential (34). Initial studies of LPS phase variation focused on possible alteration of plasmid-encoded genes. However, cloning and sequencing of the entire QpH1 plasmid (45) and plasmid sequences integrated into the chromosome of plasmidless *C. burnetii* Scurry Q217 (51) revealed no evidence for genes involved in phase variation. Furthermore, plasmid type had been correlated to the type of disease (39), but this has been refuted by several authors (44, 58). These findings suggest virulence factors to be chromosomally encoded. Restriction fragment length polymorphism (RFLP) patterns demonstrated a great heterogeneity of *C. burnetii* isolates (21, 24, 46) which may be associated with virulence potential. A physical map of *C. burnetii* Nine Mile would provide a means to

evaluate the reason(s) for this great heterogeneity and the different virulence potentials of isolates.

Due to restrictions imposed by the intracellular nature of *C. burnetii*, genetic studies have always been performed with *Escherichia coli* as the vehicle for gene expression. Nevertheless, gene organization of an organism can be studied only with an existing genetic map. Classical genetic maps are constructed by well-established methods such as transduction, transformation, conjugation, or transposon mutagenesis. But since obligate intracellular bacteria are not amenable to conventional linkage mapping, the only way to construct a genetic map—apart from sequencing—is to first establish a physical map and then locate genes on the physical map by hybridization techniques. A first step toward generation of *C. burnetii* mutants was recently undertaken by Suhan et al. (42), with the successful transformation of *C. burnetii* to ampicillin resistance. Here we present the physical and genetic map of *C. burnetii* Nine Mile constructed mainly by two methods, pulsed-field gel electrophoresis (PFGE) and PCR.

### MATERIALS AND METHODS

**Bacterial strains.** *C. burnetii* Nine Mile RSA 493 was obtained from L. P. Mallavia, Washington State University, Pullman. *C. burnetii* *NotI/Sau3a* and *NotI/EcoRI* fragments were shotgun cloned in phagemid vector pBluescript II KS(+) (Stratagene, Heidelberg, Germany) and transformed into *E. coli* XL1 Blue (Stratagene).

**PFGE sample preparation.** Heat-inactivated (15 min, 85°C) *C. burnetii* organisms were diluted to a concentration of  $2 \times 10^9$  particles/ml. One volume of this suspension was mixed with 1 volume of solubilized 1% InCert agarose (Bio-Rad, Munich, Germany) at 50°C. Agarose-embedded *C. burnetii* was lysed overnight at 56°C with proteinase K (500 µg/ml) and washed twice with 10 volumes of 1× TE (10 mM Tris-HCl, 1 mM EDTA) for 30 min. Proteinase K was inactivated by phenylmethylsulfonyl fluoride (1 mM) at 50°C. *C. burnetii* total DNA embedded in agarose plugs was digested with 10 U of *NotI*/ml in 400 µl of 2× Universal buffer (Stratagene) overnight at 37°C.

**PFGE.** All PFGE gels (1% MBC agarose; Bio-Rad) were run on a CHEF (contour-clamped homogeneous electric field) mapper apparatus (Bio-Rad). The following parameters were applied to separate *NotI* fragments of the indicated size ranges: (i) 2 to 270 kb, pulse time of 0.1 to 11.0 s for 8 h, linear gradient and then 9.0 to 24.0 s for 12 h, linear gradient at constant voltage (6 V/cm) and constant angle (120°); (ii) 50 to 270 kb, pulse time of 14.23 to 16.08 s for 23 h 22 min, linear gradient at constant voltage (6 V/cm) and constant angle (120°), ramping factor of -1.357; and (iii) 2 to 55 kb (field inversion gel electrophoresis

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[FIGE]); pulse time of 0.05 to 0.12 s for 9 h 26 min, forward voltage of 9 V/cm, reverse voltage of 6 V/cm.

**DNA probes.** Biotinylated gene probes were prepared by PCR with biotin-21-dUTP (Amersham, Braunschweig, Germany), using different dTTP/biotin-21-dUTP ratios (3:1, 6:1, and 9:1). From cloned DNA fragments, plasmid DNA was prepared and biotin labeled by nick translation (Nick translation biotinylation kit; Serva, Heidelberg, Germany).

**Southern hybridization.** To dissect large *NotI* fragments, PFGE gels were exposed to UV light (Stratalinker; Stratagene) with an energy of 60 mJ/cm<sup>2</sup>. Southern blotting was performed as downward blotting (8). Fragments were denatured (0.5 M NaOH–1.5 M NaCl, two cycles of 30 min each) and transferred to Biotodyne B membranes (Pall; Dreieich, Germany) with 20× SSC (3 M NaCl, 0.3 M sodium citrate) as the transfer buffer. Transferred DNA was immobilized by exposure to UV light (120 mJ/cm<sup>2</sup>). Hybridization experiments were carried out at 60°C with 200 ng of denatured probe. Since probed DNA was always the same (*C. burnetii* Nine Mile) and only probes differed, the membrane was cut into stripes (3 by 130 mm) and hybridizations were performed in a specially constructed hybridization chamber. Stringency washes and chemiluminescence detection of biotinylated DNA were carried out as instructed by the manufacturer (Serva).

**XL PCR.** XL (extra-large) PCR was developed for the amplification of DNA fragments of up to 40 kb (7). XL PCR was performed on a Perkin-Elmer thermal cycler (model 9600; Perkin-Elmer/ABI, Weiterstadt, Germany) in a total volume of 50 µl consisting of 1× XL buffer, 1.1 mM magnesium acetate, 0.4 µM each primer, 200 µM each deoxynucleoside triphosphate, 1 U of XL polymerase, and 10<sup>4</sup> to 10<sup>6</sup> DNA templates. To amplify the 27.5- and 11-kb fragments, the following conditions were applied (values for the 11-kb fragment are in parentheses): 15 cycles consisting of denaturation at 94°C for 15 s, annealing and extension at 68°C for 15 min and 30 s (60°C, 7 min); 15 cycles consisting of denaturation at 94°C for 15 s, annealing and extension at 68°C for 15 min and 30 s, with an autoextension time of 15 s per cycle (60°C, 7 min plus 15 s per cycle). Prior to PCR, template DNA was allowed to completely denature at 94°C for 1 min. After PCR, an additional extension step at 72°C for 10 min was applied to maintain fully double-stranded DNA.

**Sequencing.** Nonradioactive sequencing reactions were performed with a PRISM Ready Reaction Dye-Deoxy Terminator Cycle Sequencing kit (Perkin Elmer/ABI) as recommended by the manufacturer.

**Sequence analysis.** DNA sequence analysis was performed with the DNASTAR software package (DNASTAR Inc., London, England). Primers were designed with the OLIGO software program (Medprobe, Oslo, Norway) and synthesized on a model 381A DNA synthesizer (Perkin-Elmer/ABI).

## RESULTS

**Mapping strategy.** The physical map of *C. burnetii* Nine Mile has been constructed by a top-down approach in combination with PCR technology.

PFGE was applied to separate fragments after *NotI* digestion of *C. burnetii* total DNA. PFGE gels were blotted and used in Southern hybridization experiments. Simultaneously, *NotI/EcoRI* and *NotI/Sau3A* fragments respectively of *C. burnetii* were shotgun cloned and sequenced. Sequence data were analyzed for open reading frames (ORFs) containing the *NotI* restriction site. Polypeptides deduced from ORFs were compared to protein database entries. Two polypeptides showing homology to the same database entry were considered as being adjacent. Proximity was proven by PCR with total *C. burnetii* DNA as the template. Amplicons from positive PCR results were digested with *NotI* to verify the contiguity of the two fragments.

Proximity of cloned fragments without any homology to database entries was examined by checkerboard PCR. For this purpose primers deduced from sequenced fragments and directed to the *NotI* site were combined in pairs and subjected to PCR with *C. burnetii* total DNA as the template. In all other cases where adjacent fragments were missing, we applied adaptor PCR (54).

**Physical map.** PFGE has been shown to be the method of choice for constructing macrorestriction maps of bacterial genomes (10) provided that appropriate rare-cutting restriction enzymes are available. Criteria for the selection of restriction enzymes have been developed by McClelland et al. (31). Most obviously, in bacterial genomes with G+C contents above 45%, the tetranucleotide sequence CTAG is extremely rare.

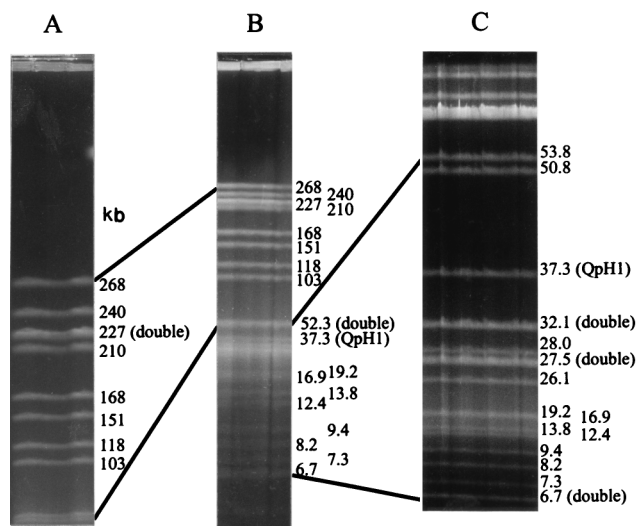


FIG. 1. CHEF-PFGE of *NotI*-digested total DNA. Different CHEF-PFGE parameters (see text) were applied to separate *NotI* fragments of 2 to 270 kb (B), 55 to 270 kb (A), and 6.7 to 55 kb (C). Lines indicate regions where resolution of *NotI* fragments has been increased. Panel A demonstrates fragments ranging in size from 210 to 268 kb to be clearly separated. Nevertheless, the two 227-kb *NotI* fragments are not distinguishable. Resolution enhancement is even more striking in panel C, where the 52.3-kb double fragment (B) divided into 53.8- and 50.8-kb *NotI* fragments. Panel C also demonstrates the 27.5- and 32.1-kb *NotI* fragments to be double fragments, as indicated by increased band intensities. In contrast, band intensity of the QpH1 plasmid suggests that there is only a single copy of the plasmid in *C. burnetii* Nine Mile.

Similarly, trinucleotides CCG and CGG are rare in genomes with G+C contents of less than 45%. The G+C content of the *C. burnetii* genome has been determined to be 43 to 45% (36). Therefore, we selected restriction enzymes *SfiI* (GGCCNN NN'NGGCC), *NotI* (GC'GGCCGC), *FseI* (GGCCGG'CC), and *SmaI* (CCC'GGG). Finally we applied restriction enzyme *NotI*, which produces 29 fragments, 25 of which are distinguishable on ethidium bromide-stained CHEF-PFGE gels (Fig. 1B). For optimal resolution, portions of the molecular weight size range where DNA fragments accumulated were expanded. Resolution of *NotI* fragments around the 200-kb region was enhanced by extending the pulse times (Fig. 1A). The 30-kb region was expanded by FIGE (Fig. 1C). The average resolution of the macrorestriction map is 72.5 kb, with 2.1 kb being the smallest and 268 kb being the largest *NotI* fragment. Altogether, the size of the *C. burnetii* chromosome is 2,103 kb (Fig. 2).

In total, 3 *NotI* fragments (2.1, 5.3, and 6.7 kb) were sequenced completely, and partial sequence information was obtained from 3 *NotI*, 59 *NotI/EcoRI*, and 15 *NotI/Sau3A* fragments. Neighbors of the 3.9-kb *NotI* fragment had already been determined (EMBL accession no. X75627 and X70045), and one neighbor of the 6.7-kb *NotI* fragment was extracted from the EMBL database (accession no. L33409). From 87 clones sequenced, 33 fragments (3 *NotI*, 28 *NotI/EcoRI*, and 2 *NotI/Sau3A*) differed in sequence. In total, 50,282 bp including those extracted from the EMBL database were analyzed. The average G+C content has been determined to be 44%. The tetranucleotide sequence CTAG occurs about 23 times less frequently (0.08%) than AAAA (1.88%), whereas trinucleotides CCG and CCG, which are parts of the *NotI* recognition site show no trend. Assuming a random distribution of nucleotides, *NotI* sites appear every 131,070 bp. Thus, statistically 16 *NotI* fragments would be generated upon restriction of the *C.*

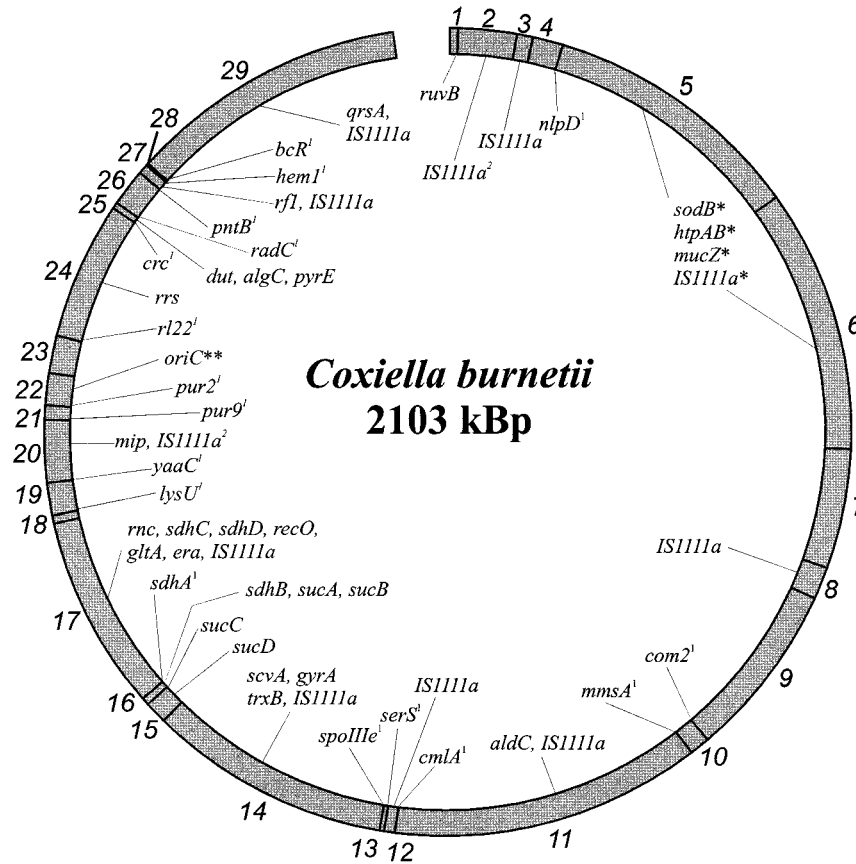


FIG. 2. Physical and genetic map of the chromosome of *C. burnetii* Nine Mile. *NotI* fragments are indicated by numbers on the outer circle (sizes in kilobases): 1, 7.3; 2, 50.8; 3, 13.8; 4, 26.1; 5, 227; 6, 227; 7, 103; 8, 27.5; 9, 151; 10, 16.9; 11, 268; 12, 9.4; 13, 3.9; 14, 210; 15, 19.2; 16, 6.7; 17, 168; 18, 6.7; 19, 28; 20, 53.8; 21, 12.4; 22, 27.5; 23, 32.1; 24, 118; 25, 5.3; 26, 32.1; 27, 8.2; 28, 2.1; 29, 240. *NotI* recognition sites are indicated by lines connecting the inner and outer circles. <sup>1</sup>, contains the *NotI* recognition site. <sup>2</sup>, hybridization signal intensity suggests *IS1111a* to exist at least once on the 50.8- and 53.8-kb *NotI* fragments (in Fig. 1B, lane 1, indicated as the 52-kb fragment). \*, Could not be related to one of the 227-kb *NotI* fragments; hybridization signal intensity suggests *IS1111a* to exist at least once on both 227-kb *NotI* fragments (Fig. 1B, lane 1). \*\*, putative genes located around the *oriC* region: *fmu*, *glvsAB*, *ygi2*, *ygi1*, *omp*, *gidB*, *gida*, 50K, 60K, and 9K.

*burnetii* chromosome (2,103 kb) with *NotI*. This is in contrast to experimental data (29 *NotI* fragments), meaning that the frequency of trinucleotides CGG and CCG is higher as expected by randomness.

We analyzed sequences for ORFs containing the *NotI* restriction site and found putative polypeptides deduced from 20 ORFs to be homologous to database entries. In each case, two polypeptides show homology to the same database entry. Hence, 10 *NotI* linking fragments (Table 1, amplicons 1, 5, 8, 13, 14, 18, 19, 23, 24, and 27) were identified simply as a result of homology to database entries. Remaining linking fragments were identified by checkerboard PCR (Table 1, amplicons 2, 4, 6, 10, 12, and 21) and adaptor PCR (Table 1, amplicons 3, 7, 9, 11, 15, 16, 22, and 25). Sequence data obtained from one adaptor fragment revealed a region containing an unusual G+C stretch of 17 bp with two nested *NotI* sites (**GCGGCCGC** GGCCGCGCC; first site in boldface and second site underlined).

Southern hybridizations were used to relate the cloned DNA fragments (*NotI/EcoRI* and *NotI/Sau3a*) to the corresponding *NotI* fragment. Nevertheless, four *NotI* fragments (227, 32, 27.5, and 6.7 kb) appeared to be double fragments. One of the 6.7-kb *NotI* fragments has been sequenced completely (EMBL accession no. X77919). The 227- and 32-kb doublets were distinguished by Southern hybridization using a PFGE blot of partially *FseI*- and *FseI/NotI*-digested total *C. burnetii* DNA.

The 27.5-kb *NotI* double fragment was differentiated by XL PCR. For this purpose, primers deduced from fragments adjacent to the 27.5-kb *NotI* fragments were combined in pairs and subjected to XL PCR with *C. burnetii* total DNA as the template. With one primer combination, we achieved a positive result with an amplicon size of about 28 kb. Hybridization with probes derived from the four DNA fragments constituting the ends of the 27.5-kb *NotI* fragments revealed positive signals with only two of them.

**Genetic map.** ORFs with homology to database entries (putative genes) and genes were located on the physical map by Southern hybridization. Hybridization with the *omp* gene as a probe revealed a hybridization signal with the 27.5-kb *NotI* double fragment. To relate the *omp* gene to one of the 27.5-kb *NotI* fragments, XL PCR was performed with primers deduced from the *omp* gene and the ends of the 27.5-kb fragments. The positive PCR result with one primer combination was confirmed by hybridization and sequencing of the 11-kb amplicon. In total, we mapped 54 genes and putative genes, of which 39 (Table 2; Table 1, amplicons 17, 20, and 26) were extracted from the EMBL database and 16 putative genes (Table 1, amplicons 1, 3, 5, 8, 10, 11, 13, 14, 16, 18, 19, 23, 24, 25, 27, and 28) were newly identified during the mapping experiments. Nineteen putative genes with homology to database entries contained the *NotI* recognition sequence. Several putative

TABLE 1. Linking fragments as identified by PCR

| Amplicon no. | Primer 1/primer 2 | Amplicon size (bp) <sup>a</sup> | EMBL accession no.  | Protein homology <sup>b</sup> (Swiss-Prot) | <i>NotI</i> linking fragments (kb) |
|--------------|-------------------|---------------------------------|---------------------|--|------------------------------------|
| 1            | 12_1/1454         | 363                             | AF064944            | PUR9_ECOLI                                 | 12.4/53.8                          |
| 2            | 1406/1408         | 240                             | AF064945            | NH   | 27.5/151                           |
| 3            | 1411/1411NB       | 328                             | AF064946            | RL22_ECOLI                                 | 32.1/118                           |
| 4            | 1412/1465         | 495                             | AF064947            | NH   | 27.5/103                           |
| 5            | 1455/516F         | 361                             | AF064948            | MMSA_PSEAE                                 | 16.9/268                           |
| 6            | 1463/5KS15        | 517                             | AF064960/X79075     | NH   | 5.3/32.1                           |
| 7            | 1467/1467NB1      | 548                             | AF064949            | NH   | 227/227                            |
| 8            | 1468/1629         | 336                             | AF064950            | YAAC_PSEFL                                 | 28/53.8                            |
| 9            | 1469/1469NB       | ≈1,800                          | AF064962/AF064963   | NH   | 19.2/210                           |
| 10           | 1473/1488         | 346                             | AF064951            | CMLA_PSEAE                                 | 9.4/268                            |
| 11           | 1474/1474NB       | ≈1,300                          | AF064964/AF064965   | NLPD_ECOLI                                 | 26.1/227                           |
| 12           | 1482/1607         | 492                             | AF064952            | NH   | 103/227                            |
| 13           | 1496/1487         | 501                             | AF064953            | RUVB_ECOLI                                 | 7.3/50.8                           |
| 14           | 1501/542NSF       | 349                             | Y10436              | GIDA_PSEPU                                 | 27.5/32.1                          |
| 15           | 1510/1510NB       | ≈1,100                          | AF064966/AF064967   | ND   | 6.7/168                            |
| 16           | 1632/1632NB       | 631                             | AF064954            | ECOLYSU                                    | 6.7/28                             |
| 17           | 200_1/4_1         | 748                             | X75627/X70045       | SP3E_BACSU                                 | 3.9/210                            |
| 18           | 20T3/1475         | 407                             | AF064955            | PUR2_ECOLI                                 | 12.4/27.5                          |
| 19           | 2KS5/1451R        | 745                             | X78969 <sup>c</sup> | HEMI_SALTY                                 | 2.1/8.2                            |
| 20           | 4_2/10_1          | 584                             | X75627/X70045       | SYS_ECOLI                                  | 3.9/9.4                            |
| 21           | 526F/1456         | 314                             | AF064956            | ND   | 13.8/26.1                          |
| 22           | 526R/526NB        | 224                             | AF064957            | NH   | 13.8/50.8                          |
| 23           | 543NSF/1452       | 200                             | AF064958            | PNTB_ECOLI                                 | 8.2/32.1                           |
| 24           | 565/1459          | 518                             | AF064959            | COM2_BACSU                                 | 16.9/151                           |
| 25           | 5T716/5T716NB     | 488                             | AF064961/X79075     | PSECR_C                                    | 5.3/118                            |
| 26           | 6KSN/6KS6307      | 591                             | L33409              | DHSA_ECOLI                                 | 6.7/168                            |
| 27           | 6SK946/1470F1R    | 1,331                           | X77919/U07789       | SUCC_ECOLI                                 | 6.7/19.2                           |
| 28           | Bicyclo/1/2       | 669                             | X78969 <sup>c</sup> | BCR_ECOLI                                  | 2.1/240                            |

<sup>a</sup> Amplicons 9, 11, and 15 have not been sequenced completely; therefore, the exact amplicon size is not known.

<sup>b</sup> From Swiss-Prot database. ORFs have been detected from amplicons 2, 4, 6, 9, and 12, but deduced polypeptides showed no homology (NH) to database entries. ORFs from amplicons 15 and 21 were not detected (ND).

<sup>c</sup> Reference 53.

polypeptides demonstrated only low homology to database entries (*bcr*, *cmlA*, and *nlpD*), though hydrophobicity plots were very similar. One gene, recognized as transposable element (23), was found to hybridize with at least nine *NotI* fragments.

16S rRNA genes appear only once on the *C. burnetii* chromosome.

The putative *oriC* of *C. burnetii* (43) is located on a 27.5-kb *NotI* fragment. Gene organization upstream the putative *oriC* of *C. burnetii* is identical to that of *Pseudomonas putida* and nearly identical to that of *Bacillus subtilis* (Fig. 3). Most strikingly, sequences downstream the *oriC* of *C. burnetii* demonstrated a gene organization unique among bacteria. The *gyrA* gene, which has been found to be clustered in some bacteria with the *dnaA*, *dnaN*, *recF*, and *gyrB* genes, has been recently identified (35) and was mapped on the 210-kb *NotI* fragment.

## DISCUSSION

Since the introduction of PFGE (40), strategies for constructing physical maps changed from bottom-up (25, 57) to top-down techniques (10). With bottom-up techniques, genome maps are established by sorting clones from a genomic library. But this procedure is tedious and may become ambiguous if one enters regions of repetitive DNA. Moreover, regions which are difficult to clone represent one of the major problems encountered in bottom-up genome mapping. In contrast, top-down approaches are easily to perform and only few restriction fragments have to be examined for their natural order. Once the physical map is established, locations of a wide variety of other restriction enzymes can be placed on the existing map with relatively little effort.

The macrorestriction map of *C. burnetii* Nine Mile has been constructed mainly by two methods, PFGE and PCR. Southern hybridization was used to relate cloned fragments to the corresponding *NotI* fragments, and PCR was used to identify linking fragments.

The size of the *C. burnetii* Nine Mile chromosome has been determined from PFGE data to be 2,103 kb and thus has been underestimated by several authors (14, 36). Myers et al. (36) used renaturation techniques to determine the chromosome size, and Frazier et al. (14) applied PFGE. Both groups calculated a size of 1,600 kb for the *C. burnetii* chromosome, which is about 25% less than found in this study. Most obviously, Frazier et al. did not recognize double fragments and/or failed to notice fragments in regions where DNA fragments accumulated. Double fragments in this study were primarily identified by increased band intensities in PFGE gels compared to single bands of similar size. Duplication of these fragments was proven by hybridization to a PFGE blot of *C. burnetii* DNA digested with a second enzyme, by partial digestion, or by XL PCR. Other mapping strategies use two-dimensional PFGE to circumvent the need for detection of double fragments. However, one general intrinsic problem with two-dimensional PFGE is the reliable detection of smaller fragments due to unfavorably low fluorescence signals of the ethidium bromide stain. Smaller fragments may even diffuse out the agarose plug during digestion.

In most cases, genomes were mapped by two different techniques whereby most often partial digests were combined with the linking clone approach. In this study, linking clones were identified by two PCR methods (checkerboard and adaptor)

TABLE 2. Genes assigned to the physical map

| Gene                     | Polypeptide                                | Accession no. <sup>a</sup> | Reference(s) |
|--------------------------|--|----------------------------|--------------|
| <i>rrs</i>               |  | M21291                     | 1            |
| <i>qrsA</i>              | Sensor-like protein                        | U07186                     | 30, 32       |
| <i>IS1111a</i>           | Transposase                                | M80806                     | 23           |
| <i>fmu<sup>b</sup></i>   | Hypothetical protein                       | U10529                     |              |
| <i>glysA<sup>b</sup></i> | Glycyl-tRNA synthetase (α chain)           | Y10435                     |              |
| <i>glysB<sup>b</sup></i> | Glycyl-tRNA synthetase (β chain)           | U10529/Y10435              | 43           |
| <i>ygi2<sup>b</sup></i>  | 32.4-kDa protein                           | Y10436                     |              |
| <i>ygi1<sup>b</sup></i>  | 28.9-kDa protein                           | Y10436                     |              |
| <i>omp (com1)</i>        | Outer membrane protein                     | Z11828/M88613              | 5, 22        |
| <i>gidB<sup>b</sup></i>  | Glucose-inhibited division protein B       | Y10436                     |              |
| <i>50K<sup>b</sup></i>   | 50-kDa protein                             | Y10436                     |              |
| <i>60K<sup>b</sup></i>   | 60-kDa protein                             | U10529                     | 43           |
| <i>9K<sup>b</sup></i>    | 9-kDa protein                              | U10529                     | 43           |
| <i>mpA<sup>b</sup></i>   | RNase P                                    | U10529                     | 43           |
| <i>rpmH<sup>b</sup></i>  | Ribosomal protein L34                      | U10529                     | 43           |
| <i>mip</i>               | Macrophage infectivity potentiator protein | U14170                     | 9, 33, 41    |
| <i>rnC</i>               | RNase III                                  | L27436                     | 60           |
| <i>recO</i>              | Repair and recombination protein           | L27436                     | 60           |
| <i>era</i>               | GTP-binding protein                        | L27436                     | 60           |
| <i>sdhB</i>              | Succinate dehydrogenase (small subunit)    | X77919                     | 20           |
| <i>sdhC<sup>b</sup></i>  | Succinate dehydrogenase                    | L33409                     |              |
| <i>sdhD</i>              | Succinate dehydrogenase                    | X77919                     | 20           |
| <i>gltA</i>              | Citrate synthase                           | M36338                     | 16, 19       |
| <i>sucA<sup>b</sup></i>  | 2-Oxoglutarate dehydrogenase               | X77919                     |              |
| <i>sucB<sup>b</sup></i>  | Dihydrolipoamide succinyltransferase       | X77919                     |              |
| <i>sucD<sup>b</sup></i>  | Succinyl-coenzyme A synthetase             | X77919                     |              |
| <i>scvA</i>              | Small cell variant protein                 | L49019                     | 18           |
| <i>trxB<sup>b</sup></i>  | Thioredoxin reductase                      | X75627                     |              |
| <i>gyrA<sup>b</sup></i>  | DNA gyrase (subunit A)                     | S82903                     | 35           |
| <i>aldC<sup>b</sup></i>  | α-Acetolactate decarboxylase               | U14393                     |              |
| <i>sodB</i>              | Superoxide dismutase                       | M74242                     | 17           |
| <i>htpAB</i>             | Heat shock proteins                        | M20482                     | 49           |
| <i>mucZ</i>              | Mucooidy-inducing protein                  | L42518                     | 59           |
| <i>dut<sup>b</sup></i>   | dUTP nucleotide hydrolase                  | X79075                     |              |
| <i>algC</i>              | Phosphomannomutase                         | X79075                     |              |
| <i>pyrE<sup>b</sup></i>  | Orotate phosphoribosyltransferase          | X79075                     |              |

<sup>a</sup> EMBL/GenBank database entry.

<sup>b</sup> Putative gene for which there is no experimental evidence; designation corresponds to homologous genes in the database.

instead of screening conventional DNA libraries or applying cross-hybridization techniques. The PCR methods circumvent some of the problems associated with cloned linking fragments. For instance, if two unlinked *NotI*-containing fragments are coligated, aberrant linking clones can be obtained. Cross-hybridization techniques use gel-purified probes to identify linking fragments. Nevertheless, gel-excised fragments contain impurities of randomly sheared DNA, leading to increased background hybridization signals. This increased background may hamper the unambiguous detection of faint hybridization signals generated from probes with very short overlaps. Moreover, in regions where DNA fragments accumulate, it is almost impossible to excise gel plugs without contamination with neighboring bands. Another restriction may be the amount of DNA in this fragment required to produce a reliable probe. A prerequisite to identify linking clones by PCR methods is the availability of sequences to construct primers. Sequences herein were obtained from shotgun cloning experiments. To make sure that clonable sizes are generated after digestion of *C. burnetii* total DNA, we chose as the second restriction enzyme (apart from *EcoRI*) *Sau3A*, which produces DNA fragments below 2 kb in size.

It remains uncertain whether the *C. burnetii* chromosome is linear or circular. However, several experimental results indicate the presence of a linear chromosome. Thus, it was not possible to clone the ends of the two fragments which would

constitute the right and left ends of the linear chromosome, nor was it possible to amplify one of the putative ends (the 7.3-kb fragment) by XL adaptor PCR. In addition, undigested *C. burnetii* total DNA migrates as a single band on PFGE gels (data not shown), whereas open circular DNA molecules larger than 15 kb fail to migrate in PFGE (28). To date, linear chromosomes have been found in *Borrelia burgdorferi* (13), *Streptomyces lividans* (27), *Agrobacterium tumefaciens* (3), and *Rhodococcus fascians* (11), but nothing is known about the mechanism of replication in bacteria with linear chromosomes.

The *oriC* of *C. burnetii* has not yet been determined unequivocally. Gene organization around the *oriC* varies according to the taxonomic classification of the organism. Nevertheless, certain bacterial genes involved in replication are clustered. Whereas in all other organisms investigated so far, genes *dnaA*, *dnaN*, and *gyrB* are clustered and in the vicinity of *rpmH*, none of these genes is present downstream the *oriC* of *C. burnetii* (Fig. 3). This unusual gene organization reflects a replication mechanism quite different from that observed in bacteria with circular chromosomes. Therefore, further investigations are feasible to characterize the nature of the ends (e.g., single-stranded loops and telomeric structure) of the linear chromosome. The *gyrA* gene occupies a central position on the physical map of *C. burnetii* Nine Mile as has also been demonstrated for the *gyrA* genes of, for example, *Borrelia burgdorferi*, *Clostridium perfringens*, *B. subtilis*, and *Leptospira inter-*

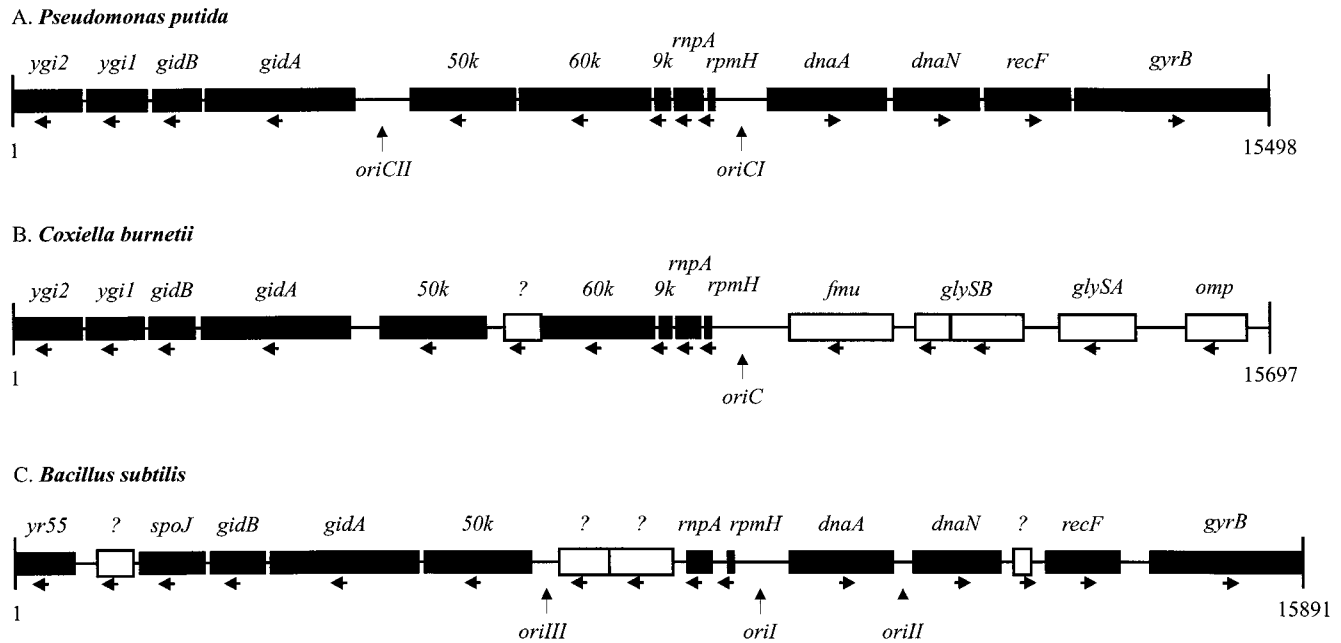


FIG. 3. Gene organization around the putative *oriC* of *C. burnetii* (B) compared to that of *P. putida* (A) and *B. subtilis* (C). The region upstream the *oriC* is almost identical to that of *P. putida* and *B. subtilis* (indicated by black boxes). Hypothetical proteins YGI1 and YGI2 of *C. burnetii* are homologous to YR55 and SpoJ of *B. subtilis*. Gene organization downstream the putative *oriC* of *C. burnetii* is quite different from that of *P. putida* and *B. subtilis*, as indicated by white boxes. Whereas in *P. putida* and *B. subtilis* genes around the *oriC* are transcribed in opposite direction, genes in *C. burnetii* are all transcribed in the same direction (indicated by arrows). Question marks above the white boxes indicate polypeptides without designation and with unknown function.

*rogans* (10). Nevertheless, presence of the *dnaA* gene, which is essential for replication and which is clustered with the *gyrA* gene in above-mentioned bacteria, remains to be confirmed.

The genetic map of *C. burnetii* revealed one gene which is recognized as a transposable element to be evenly distributed over the whole chromosome. Hoover et al. (23) demonstrated IS1111a to be present at least 19 times on the *C. burnetii* Nine Mile chromosome. We have mapped nine copies of the IS1111a gene, indicating that the gene appears more than once on several *NotI* fragments. Primers derived from the IS1111a element were used to establish a diagnostic PCR (52). House-keeping genes belonging to the Krebs cycle show gene organizations identical to that of, for example, *E. coli* (12) or *Azotobacter vinelandii* (50).

On the *C. burnetii* chromosome there is only a single copy of the 16S rRNA gene, located on the 118-kb *NotI* fragment. This finding is in agreement with results published by Afseth and Mallavia (1) and correlates with the slow growth rate of *C. burnetii*. Though no direct evidence relates the number of *rnm* operons in an organism to growth rate regulation, slowly growing organisms such as *Spiroplasma citri* (56), *Mycoplasma pneumoniae* (26), and *Chlamydia trachomatis* (4) have been shown to have fewer copies.

As has been shown by DNA solution hybridization (29), *C. burnetii* isolates have many commonalities. However, RFLP analysis demonstrated a great heterogeneity in restriction patterns (21, 24, 46), possibly due to missense mutations and/or restriction site redistributions. Redistributions again are a result of significant chromosomal rearrangements (e.g., translocations, inversions, insertions, or deletions) which may play an important role in pathogenesis or virulence (47, 48). Similar rearrangements and exchange of DNA blocks have been described for *Pseudomonas aeruginosa* (38), leading to a 10% variation in chromosome size. Variation in chromosome size is even more striking in *C. burnetii* isolates ranging from about

1,500 to 2,400 kb. The high degree of DNA homology among *C. burnetii* isolates suggests that deletions resulting from recombinational (homologous or heterologous) events generated smaller genomes from larger ones. The now existing library of *NotI* linking fragments will facilitate comparisons of genome organization of different isolates and thus help to elucidate the reason(s) for the great heterogeneity in RFLP patterns. Furthermore, linking fragments may help to clarify which parts of the chromosome are the most conserved or variable ones and may help to answer the question of whether smaller *C. burnetii* genomes resulted from deletions in larger genomes. If so, this would provide a means for extensive epidemiological studies to evaluate the ubiquitous abundance of *C. burnetii*. The genetic map of *C. burnetii* Nine Mile may serve as a basis to identify genes or gene clusters affected by this tremendous loss of genetic information which seems to be no longer necessary for the organism to survive. It has been shown for *Borrelia* species that gene order is conserved, though nearly all isolates investigated demonstrated unique RFLP patterns (6). Whether this is also true for *C. burnetii* will be evaluated through comparison of gene order between isolates with different RFLP patterns.

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