Plasmid-Homologous Sequences in the Chromosome of Plasmidless *Coxiella burnetii* Scurry Q217

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Chromosomal DNA from *Coxiella burnetii* Scurry Q217 was screened for the presence of plasmid-homologous sequences. Total DNA from Scurry Q217 was digested with *NotI*, and the resulting DNA fragments were separated by contour-clamped homogeneous electric field pulsed-field gel electrophoresis (CHEF-PFGE). Following hybridization with biotin-labeled QpH1 plasmid as a probe, two DNA fragments of 40 and 170 kb were identified as targets. These fragments were cloned, and subclones containing QpH1-homologous sequences were completely sequenced. The physical mapping of DNA fragments was achieved by PCR with primers derived from adjacent fragments, and a total of 18,360 bp was sequenced. Within the QpH1-homologous region spanning 16,624 bp, homology was as high as 99%. Deletions were identified within *EcoRI* fragments A^H-C^H-K^H-B^H (13,490 bp) and J^H-G^H-E^H-L+-D^H (6,509 bp) and in fragment A^H alone (619 bp). An insertion of 744 bp was identified within the JD^c region of Scurry Q217. A search for putative coding regions identified a total of 17 open reading frames (ORFs). Compared to plasmid QpH1, 6 ORFs were identical, 5 ORFs were different in size, 6 ORFs were newly generated, and 25 ORFs were lost. It was found that plasmid-homologous sequences in Scurry Q217 were of chromosomal origin.

Coxiella burnetii, the only member of the genus *Coxiella*, is the causative agent of Q fever in humans as well as of abortions in domestic animals, especially ruminants. So far, different criteria have been applied for the classification of *C. burnetii* isolates. Samuel et al. (7) distinguish between so-called acute and chronic isolates that cause acute or chronic Q fever in humans, apparently depending on the plasmid type. However, recent experimental findings (3) and PCR analysis of *C. burnetii* strains isolated from patients exhibiting chronic Q fever (11) revealed that isolates containing either QpH1 or QpRS can cause endocarditis. Yu and Raoult (15) confirmed these results by serotyping isolates with monoclonal antibodies and postulated that the development of acute or chronic Q fever depends on the patient's condition and immune status rather than on the plasmid type of the causative agent. Nevertheless, plasmids are still considered to be of major importance concerning virulence factors, mechanisms of pathogenicity, and proliferation in the phagolysosomal compartment.

Assuming that the plasmid-coded sequences are of essential importance for *C. burnetii*, the existence of the plasmidless *C. burnetii* isolate Scurry Q217 (8) was rather unexpected. However, it was shown that Scurry Q217 contains chromosomally integrated plasmid-homologous DNA sequences (8). In an attempt to understand the significance of plasmids in *C. burnetii*, we examined plasmidless isolates with chromosomally integrated plasmid-homologous DNA fragments.

MATERIALS AND METHODS

DNA-modifying enzymes. Restriction endonucleases and other DNA-modifying enzymes were purchased from Stratagene (Heidelberg, Germany) and Amersham (Bad Homburg, Germany) and used under conditions recommended by the manufacturers.

Plasmids and bacterial strains. *C. burnetii* DNA fragments were cloned in phagemid vector pBluescript II KS(+), cosmid vector SuperCos 1, or lambda phage vector EMBL4 (Stratagene). Recombinant cosmid and lambda phage DNAs were packaged (Gigapack II packaging extract; Stratagene) and transfected into *Escherichia coli* XL1 Blue (Stratagene). *C. burnetii* reference isolates Scurry Q217, Priscilla Q177, Dugway 7E22-57, and Nine Mile RSA493 were obtained from L. P. Mallavia, Washington State University, Pullman, Washington.

PFGE. Contour-clamped homogeneous electric field pulsed-field gel electrophoresis (CHEF-PFGE) was performed with the CHEF Mapper apparatus (Bio-Rad, Munich, Germany). InCert agarose (Biozym, Hessisch Oldendorf, Germany) plugs containing 5×10^7 *C. burnetii* particles were lysed overnight by proteinase K (final concentration, 500 µg/ml) treatment and digested with *NotI* (4 U/plug). Conditions to separate the resulting DNA fragments on a 1% agarose (molecular biology certified) (Bio-Rad) gel were as follows: 170 V for 8 h with a pulse time of 0.5 to 28 s and, subsequently, 170 V for 8 h with a pulse time of 8 s. To separate DNA fragments ranging from 150 to 200 kb on a low-melting-point agarose gel, the following conditions were applied: total time, 20.5 h; pulse time, 12.26 to 15.33 s; ramping factor, -1.364.

DNA hybridizations. DNA probes were biotinylated with a commercial nick translation biotinylation kit (Serva, Heidelberg, Germany). After depurination of the large DNA fragments (0.25 N HCl; 2 cycles of 15 min per cycle) and denaturation (0.5 N NaOH, 1.5 N NaCl; 2 cycles of 30 min per cycle), DNA fragments were downward blotted (1) on Biotyne B (Pall, Dreieich, Germany) nylon membranes with $20\times$ SSC (3 M NaCl, 0.3 M sodium citrate) as the transfer buffer. Hybridizations were carried out at 60°C overnight with the Southern Light hybridization kit (Serva).

Plaque lifts. Biotyne A (Pall) nylon membranes were placed for 2 min on precooled (4°C) petri dishes (90 mm in diameter) with lambda phage plaques and were asymmetrically marked. The membranes were removed from the petri dishes, and the transferred DNA was denatured (0.5 N NaOH, 1.5 N NaCl; 2 min) and neutralized (0.5 M Tris-HCl [pH 7.4], 1.5 N NaCl; 2 min). Subsequently, the membranes were air dried and the DNA was immobilized by UV irradiation (120 mJ/cm²). Hybridization was performed at 60°C with the Southern Light hybridization kit.

Sequencing primers. Primer sequences were derived from newly obtained sequence data (primer walking sequencing strategy) and designed with the aid of the Oligo primer analysis software package (MedProbe, Oslo, Norway). Primers were synthesized on a DNA synthesizer 381 A (Perkin-Elmer/ABI, Weiterstadt, Germany). Chemicals were purchased from Roth (Karlsruhe, Germany) and MWG (Ebersberg, Germany). Primers were used for sequencing without further purification.

HPLC purification. To remove primers and nucleotides, PCR amplicons were high-performance liquid chromatography (HPLC) purified by ion exchange chromatography (Gen Pak-Fax ion exchange column; Millipore, Eschborn, Germany) with the following salt gradient: 0 to 9 min, 50% buffer 1 (25 mM Tris-HCl [pH 7.5]); 9 to 10 min, 50 to 55% buffer 2 (25 mM Tris-HCl [pH 7.5], 1 M NaCl), 10

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FIG. 1. (A) *NotI*-digested total DNA of *C. burnetii* after CHEF-PFGE; (B) DNA-DNA hybridization with biotin-labeled QpH1 A fragment in pBluescript II KS(+) vector (pBluescript II KS(+) vector hybridizes with 5-kb ladder) as probe. Lane 1, 5-kb ladder; lane 2, Nine Mile RSA493; lane 3, Scurry Q217.

to 15 min, 55% buffer 2; 15 to 16 min, 55 to 57% buffer 2; 16 to 21 min, 57% buffer 2. Fractions of 360 μ l were collected. DNA in the fraction containing the amplicon was ethanol precipitated by adding 40 μ l of 3 M sodium acetate (pH 7.0) and 1 ml of 99% ethanol.

Sequencing. Nonradioactive sequencing reactions were performed with the PRISM ready-reaction dye-deoxy terminator cycle sequencing kit (Perkin-Elmer/ABI) as recommended by the manufacturer.

DNA sequence analysis. DNA sequence analysis was performed with the DNASTAR software package (DNASTAR Inc., London, United Kingdom).

PCR. PCR was performed on a Perkin-Elmer thermal cycler (model 9600) to map Scurry Q217 plasmid-homologous *EcoRI* fragments at the following conditions: 35 cycles consisting of denaturation at 95°C for 15 s, annealing at 50 to 55°C (depending on the melting temperature of the primers) for 15 s, and extension at 72°C for 60 s.

Nucleotide sequence accession number. The sequence obtained in this study has been assigned GenBank accession number X93204.

RESULTS

Detection of chromosome-integrated plasmid sequences of *C. burnetii* isolate Scurry Q217. Genomic DNA of *C. burnetii* isolate Scurry Q217 was digested with *NotI*, and the resulting fragments were separated by CHEF-PFGE (Fig. 1A, lane 3). The presence of plasmid-homologous sequences was detected by Southern hybridization with biotin-labeled QpH1 A fragment as the probe. Two hybridization signals corresponding to the 40- and 170-kb *NotI* fragments of *C. burnetii* isolate Scurry Q217 were detected (Fig. 1B, lane 3).

Cloning and subcloning of the 40-kb *NotI* fragment. Total Scurry Q217 genomic DNA was restricted with *NotI* and was shotgun cloned into SuperCos 1. Recombinants were examined for plasmid-homologous sequences by hybridization with biotin-labeled QpH1 plasmid. DNA of positive clones was digested with *EcoRI*, and hybridization with QpH1 as the probe revealed signals for six fragments. Five of these fragments were subcloned into a pBluescript II KS(+) vector as *NotI*-*EcoRI* (Fig. 2A, lane 4) and *EcoRI*-*EcoRI* (Fig. 2A, lanes 5, 6, 8, and 9) fragments. Recombinants were verified by hybridization with QpH1 as the probe (Fig. 2B, lanes 4 to 6, 8, and 9) and sequenced. After the mapping of the *EcoRI* fragments (see below) the missing H^c (the superscript "c" denotes chromosomal fragments of Scurry Q217) fragment was PCR amplified (Fig. 2A, lane 7) with primers derived from adjacent *EcoRI* fragments. The amplicon was HPLC purified and sequenced.

Plasmid-homologous *EcoRI* fragments of Scurry Q217 were labeled according to their homology to QpH1 *EcoRI* fragments.

Identification and cloning of QpH1-homologous sequences of the 170-kb *NotI* fragment. Scurry Q217 total DNA was *NotI* restricted, and fragments were separated by CHEF-PFGE. The 170-kb *NotI* fragment was excised from the gel and was in-gel digested with *EcoRI*. The resulting fragments were separated by conventional agarose gel electrophoresis. To evaluate the sizes of plasmid-homologous sequences, the DNA fragments were blotted and hybridized with QpH1 and the A fragment of QpH1 as probes. Both hybridizations showed only one signal with a 12-kb *NotI*-*EcoRI* fragment (data not shown), indicating that the 170-kb *NotI* fragment contained only sequences with homology to the QpH1 A fragment.

Therefore, Scurry Q217 total DNA was digested with *EcoRI* and was shotgun cloned into the vector lambda EMBL4. Recombinants were localized by plaque hybridization with labeled QpH1 A fragment as the probe. Recombinant DNA was subcloned as *NotI*-*EcoRI* (Fig. 2A, lane 2) and *NotI*-*XhoI* (Fig. 2A, lane 3) fragments into a pBluescript II KS(+) vector, verified by hybridization (Fig. 2B, lane 2 and 3), and sequenced.

Mapping of Scurry Q217 plasmid-homologous *EcoRI* fragments by PCR (Fig. 3). The arrangement of the QpH1 *EcoRI* fragments has already been published (12) and was used as the basis for the mapping of the Scurry Q217 *EcoRI* fragments by PCR. Primers derived from putatively adjacent *EcoRI* fragments were directed to the corresponding *EcoRI* site. The

FIG. 2. (A) Restriction analysis of pBluescript II KS(+) clones; (B) hybridization with biotin-labeled QpH1 probe in cosmid vector (cosmid vector DNA hybridizes with pBluescript II KS(+) vector DNA). Lanes: 1, lambda DNA, *HindIII* fragment; 2, clone 5/1, *NotI*-*EcoRI* fragment; 3, clone 4/3, *NotI*-*XhoI* fragment; 4, clone 6/19, *EcoRI*-*NotI* fragment; 5, clone 2/8, *EcoRI*-*EcoRI* fragment (JD^c fragment); 6, clone 1/29, *EcoRI*-*EcoRI* fragment (F^c fragment); 7, PCR amplicon (H^c fragment); 8, clone 2/1, *EcoRI*-*EcoRI* fragment (B^c fragment); 9, clone 2/13, *EcoRI*-*EcoRI* fragment (I^c fragment); 10, pBluescript II KS(+) vector, *EcoRI* fragment; 11, lambda DNA, *HindIII*-*EcoRI* fragment.

FIG. 3. Restriction map of the Scurry Q217 plasmid-homologous chromosomal region. The designations of pBluescript II KS(+) clones are shown as numbers in the *Eco*RI restriction map. E, *Eco*RI; N, *Not*I; X, *Xho*I; PCR, PCR product. Numbers above the *Bam*HI, *Hpa*I, and *Bgl*II restriction fragments indicate their sizes in base pairs (in parentheses are published data from Savinelli and Mallavia [8]). Arrowheads mark the beginning and the end of plasmid-homologous sequences. Dashed lines represent chromosomal sequences.

correctness of the amplicon was verified by restriction with *Eco*RI and by sequencing. The QpH1-homologous M^c fragment consisting of two directly adjacent *Eco*RI sites could also be detected in Scurry Q217 upon sequencing the respective PCR product.

Comparison of Scurry Q217 plasmid-homologous sequences with QpH1 plasmid sequences (Fig. 4, Table 1). *Eco*RI fragments A^c, B^c, D^c, F^c, H^c, I^c, J^c, and M^c span a region of 16,624 bp and show homologies to corresponding sequences of the QpH1 plasmid. The deletion of C^H- and K^H-homologous fragments in Scurry Q217 has been demonstrated by hybridization.

A comparison with the A^H-C^H-K^H-B^H region of QpH1 revealed a deletion of 13,490 bp in Scurry Q217. Additionally 619 bp were deleted in the A^c fragment. Another deletion covers fragments G^H, E^H, and L^H as well as parts of the J^H and D^H fragments. Homology of the JD^c fragment to parts of the J^H and D^H fragments is interrupted by an insertion of 744 bp of non-QpH1 homologous sequences.

Sequence data analysis. Chromosomal sequence data from *C. burnetii* isolate Scurry Q217 comprise 18,360 bp of double-stranded DNA with a GC content of 39.2 mol% (QpH1 plasmid: 39.3 mol% [10]).

FIG. 4. Chromosomal arrangement of QpH1 (B) and of homologous plasmid sequences in Scurry Q217 (A). ORFs are indicated by boxes and numbers (black boxes, 100% homology; grey boxes, ORF alterations due to deletions or insertions; open boxes, ORFs unique to Scurry Q217). ORF 1 (not included in this map) is located in the chromosomal region upstream to the B^c fragment. The transcription direction is indicated by arrows. Downward vertical lines indicate *Eco*RI sites (for designation of *Eco*RI fragments see text); dashed lines represent deletions in Scurry Q217 compared to QpH1. Symbols: X, *Xho*I; N, *Not*I; I, insertion of 744 bp; c, chromosomal sequences.

TABLE 1. QpH1-homologous *Eco*RI fragments of *C. burnetii* isolate Scurry Q217

Fragment ^a	<i>Eco</i> RI fragment cloning in:	Length (bp)	Homology to QpH1 (bp)
A ^c	EMBL4	~17,000	11,806
B ^c	BS ^b	929	433
JD ^c	BS	1,883	525 (D ^c) 662 (J ^c)
F ^c	BS	1,708	1,706
H ^c	BS	886	875
I ^c	BS	684	687

^a The A^c fragment was subcloned into pBluescript II KS(+) as *NotI*-*XhoI* (5,882 bp, clone 4/3) and *NotI*-*Eco*RI (approximately 11 kb, clone 5/1) fragments (Fig. 2A, lane 2; Fig. 2B, lane 2).

^b BS, pBluescript II KS(+).

Sequence data were compared to database entries (EMBL 43, GenBank 90, SwissProt release 31) but only homologies to *C. burnetii* plasmid entries were found. An analysis of sequence data for protein-coding sequences revealed 17 open reading frames (ORFs) (Fig. 4) consisting of ≥ 300 bp (100 amino acids) compared to 37 ORFs for the QpH1 plasmid. Six of the 17 ORFs were identical to those of the QpH1 plasmid, whereas 5 ORFs were altered (insertion or deletion) and 6 ORFs were unique to Scurry Q217 (Fig. 4). ORFs 4 and 11 as well as ORFs 5 and 10 showed significant homologies (63.7 and 58%, respectively) to each other. Further sequence homologies to QpDV plasmid entries (accession numbers, X84722, X85011, and X85964) exist. No homology to the only published QpRS plasmid sequence (accession number, M29982) could be detected.

Determination of the chromosomal integration site. At both ends of the investigated Scurry Q217 sequence, regions were identified showing no homology to QpH1 or to other published plasmid sequence data. To confirm that these sequences were of chromosomal origin, PCR was performed with primers derived from non-plasmid-homologous regions of Scurry Q217 (primer 1, 65 bp upstream of the integration site; primer 2, 233 bp downstream of the integration site). The PCR results were positive (amplicon size, 298 bp) for all isolates tested except for Scurry Q217 (Fig. 5). These findings indicate that all isolates contain sequences homologous to Scurry Q217 chromosomal DNA. This was further proved by the sequencing of the amplicons from different isolates carrying different plasmid types and by the multiple alignment of these sequences. Primers in the plasmidless isolate Scurry Q217 flank the entire chromosome-integrated plasmid sequence, which is about 16 kb long. Consequently, no amplicon could be produced (Fig. 5, lanes 5 and 6) when PCR conditions for amplification of short DNA fragments were used.

DISCUSSION

The detection of chromosomally integrated plasmid-homologous DNA fragments in *C. burnetii* isolate Scurry Q217 by hybridization experiments (8) suggests that this genetic information is of crucial importance for the organism. Unlike in other bacteria, where plasmid genes are believed to provide supplementary activity (7), plasmid sequences are supposed to have a critical function in *C. burnetii*, since they have been conserved in all isolates examined (7), even in the plasmidless isolate Scurry Q217.

PFGE. Several groups have applied the PFGE method to differentiate *C. burnetii* isolates (2, 4, 13) and to screen for plasmid-homologous sequences (4). Heinzen et al. (4) and Frazier et al. (2) demonstrated that after PFGE, *Sfi*I and *Not*I

fragment patterns of total DNA of the plasmidless isolates Scurry Q217, L Q216, G Q212, and Ko Q229 are identical. Hybridization experiments with ³²P-labeled QpH1 showed signals with two *Not*I fragments (4); the results of those experiments were confirmed in this study (Fig. 1B).

Restriction map. A restriction map of plasmid-homologous and adjacent chromosomal regions of the plasmidless *C. burnetii* isolate Ko has been published by Savinelli and Mallavia (8). The authors used total genomic DNA of *C. burnetii* isolate Ko restricted with *Eco*RI to demonstrate the existence of plasmid-homologous sequences. Hybridization with ³²P-labeled QpRS or ³²P-labeled QpH1 plasmid revealed six bands of homology in each case. From a chromosomal gene bank of *C. burnetii* isolate Ko, they have isolated a cosmid clone bearing the whole plasmid-homologous region. Subsequently, the arrangement of the *Eco*RI fragments was determined by mapping the cosmid clone with different restriction enzymes (*Bam*HI, *Hpa*I, *Bgl*I, and *Bgl*II). Figure 3 demonstrates the restriction map of the Scurry Q217 plasmid-homologous chromosomal region as derived from the present study. Compared to the published data of Mallavia and Samuel (5), there is one significant difference in the arrangement of fragments after restriction with *Bam*HI: whereas in our data the first and second *Bam*HI fragments consist of 2,232 and 4,381 bp, respectively, the fragment sizes in the data of Mallavia and Samuel are 4,630 and 2,180 bp, respectively. Most probably, these two *Bam*HI fragments have been reversed in their order. There is no restriction site for *Bgl*I in the plasmid-homologous sequences. This finding, as well as the restriction maps for *Eco*RI, *Bgl*II, and *Hpa*I, is in accordance with our data (Fig. 3).

Samuel et al. (7) demonstrated homology among *Eco*RI fragments of *C. burnetii* plasmids (QpH1 and QpRS) and *Eco*RI fragments generated from the plasmid-homologous region of the plasmidless isolate Ko. An *Eco*RI restriction map of the plasmid-homologous region of isolate Ko has been previously published by Savinelli and Mallavia (8). Taking the map of Savinelli and Mallavia (8) as a basis, the results of Samuel et al. (7) are in good agreement with our results except for two differences: Samuel et al. describe homologies between JD^c and D^H+I^H fragments as well as between I^c and J^H fragments, whereas in the present study JD^c and J^H+D^H as well as I^c and I^H are homologous (Fig. 4).

The core region. Cross-hybridization experiments with plasmids QpRS, QpDG, and QpH1 demonstrated large regions of significant homology subsequently termed the core regions (5). The authors determined that these regions encompassed 25 kb and that they showed homology also to chromosomal se-

FIG. 5. Determination of the integration site by PCR with total DNA of the plasmidless isolate Scurry Q217 (lanes 5 and 6) and of plasmid-bearing isolates Nine Mile RSA493 (lanes 1 and 2), Priscilla Q177 (lanes 3 and 4), and Dugway 7E22-57 (lanes 7 and 8) as templates. Marker, 100-bp ladder (lane 9); negative control, lane 10.

quences of plasmidless isolates (5, 14). However, recent results of Thiele et al. (12) as well as the present study demonstrated a homology of Scurry Q217 plasmid-homologous sequences of more than 99% in a region of only 16.6 kb. Comparing ORFs derived from the chromosome-integrated plasmid sequences with those from the QpH1 plasmid revealed that 6 ORFs were conserved. The putative polypeptides showed no homology to any entry in protein databases.

Mutations. Compared to those of QpH1, most of the deleted ORFs carry genetic information for proteins that are putatively involved in the replication of plasmids (termed replication proteins, DNA helicase I, or SOPA proteins) and that are no longer necessary for the replication of the plasmidless Scurry Q217 DNA. Surprisingly, sequences encoding a protein with homology to the ferripyochilin binding protein (FBP), considered to be a virulence factor of *Pseudomonas aeruginosa*, were also deleted in Scurry Q217. FBP plays an important role as a siderophore receptor in the regulation of Fe²⁺ metabolism. The deletion has been confirmed by negative results in dot blot and PCR experiments (10). Other mutations in the plasmid-homologous sequences of Scurry Q217 affect intergenic regions.

Interestingly, the deletion of a 619-bp fragment from the A^c fragment of Scurry Q217 seems to be accomplished by reciprocal recombination between direct repeats. Direct repeats (GGCCCAATTCGTC) have been found in the QpH1 plasmid at position 27554 directly upstream to the deletion site (position 27567) and at the 3' end of the deleted sequence (position 28172). The sequence of the direct repeat in the plasmid-homologous sequences of Scurry Q217 is located at position 11969. During reciprocal recombination the 619-bp fragment and one of the repeats were excised in Scurry Q217 plasmid-homologous sequences.

Chromosomal integration site. So far, no information is available on the chromosomal integration site of chromosomally integrated plasmid-homologous sequences of Scurry Q217. An analysis of sequence data revealed one motif (TTCCTTT) found in the chromosomal region of Scurry Q217 as well as in chromosomal DNA of plasmid-bearing isolates (Nine Mile RSA493, Priscilla Q177, and Dugway 7E22-57) upstream of the integration site. Nearly the same motif (TTCCTAT) has been found in the QpH1 plasmid right before the beginning of Scurry Q217 homologous sequences. Plasmid and chromosomal sequences probably fused via this motif. Subsequently, plasmid sequences may have been integrated into the chromosome by deletion of the TTCCTAT sequence.

Future aspects. So far, all *C. burnetii* isolates have been found to contain plasmids or plasmid sequences integrated into the chromosome. Although the biological significance of *C. burnetii* plasmids is still unknown, we suggest that plasmid sequences carry essential factors and/or perform essential functions for the organism. The functions of these conserved regions can only be evaluated if transformation of *C. burnetii* isolates is possible and deletion mutants become available. The success of Suhan et al. (9) in transforming *C. burnetii* indicates that such analysis is feasible.

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