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Physical Map of the *Bartonella bacilliformis* Genome

CHARLES M. KRUEGER, KATHERINE L. MARKS, AND GARRET M. IHLER*

Department of Medical Biochemistry and Genetics, College of Medicine, Texas A&M University Health Science Center, College Station, Texas 77843-1114

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The genome of *Bartonella bacilliformis* was shown to be a single circular DNA molecule of about 1,600 kbp having six *Not*I, four *Sfi*I, and two *Ceu*I sites. A physical map of the DNA was constructed by contour-clamped homogeneous electric field pulsed-field gel electrophoresis of DNA restriction fragments. rRNA operons, the invasion-associated locus, and a flagellin gene were located on the map by hybridization.

Bartonella bacilliformis is a flagellated, highly motile, hemotrophic, gram-negative bacterium (6, 13) which is transmitted to humans by sand fly bites (4, 6, 7, 13). It is the causative agent of Carrión's disease, an endemic disease in certain areas of Columbia, Ecuador, Peru, Bolivia, and Chile (4). To facilitate study of this interesting bacterium, we have constructed an initial physical map of its DNA by pulsed-field gel electrophoresis (PFGE) of *Not*I, *Sfi*I, and *Ceu*I restriction fragments.

Number and sizes of restriction fragments. *B. bacilliformis*, grown to 1×10^8 to 2×10^8 bacteria per ml as previously described (9), was washed with phosphate-buffered saline (PBS), resuspended in 1 ml of PBS, mixed with 1 ml of molten 2% pulsed-field agarose (Bio-Rad Laboratories, Richmond, Calif.), and poured into a commercial plastic mold (Bio-Rad, Richmond, Calif.) designed to form thin agarose slabs. The bacteria were lysed within the agarose slabs by soaking the slabs in 1% sodium dodecyl sulfate–proteinase K (1 mg/ml)–TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA [pH 8.0]) for 48 h at 37°C, rinsing them with 10 changes of TBE, soaking them in 100% ice-cold acetone for 20 min, and rinsing them with 5 changes of TBE. The processed slabs were stored in 0.5 M EDTA at 4°C. Digestions were performed on small agarose plugs (9 by 1 by 1 mm) cut from the slabs. The plugs were washed and placed into microcentrifuge tubes. Restriction endonuclease digestions were performed under conditions recommended by New England Biolabs (Beverly, Mass.). After electrophoresis, the DNA was initially visualized with ethidium bromide. In later experiments, ^{32}P labeling of DNA within the agarose slabs was accomplished by including dATP, dTTP, dGTP (25 nM each), [^{32}P]dCTP (25 μCi/ml), and *Taq* polymerase (12.5 U/ml) in the restriction digestion reaction mixtures; unincorporated radioactive material was removed by washing before electrophoresis. After electrophoresis, the DNA was stained with ethidium bromide, photographed, nicked, vacuum transferred to a nylon membrane, and autoradiographed.

Digestion with *Sfi*I produced four DNA fragments, S1 to S4 (Fig. 1A, lane 3), with sizes of 753, 602, 247, and <50 kbp, respectively, measured by PFGE with a CHEF-DRII or -DRIII apparatus (Bio-Rad, Hercules, Calif.). By standard gel electrophoresis, the size of the fourth band was shown to be 15 kbp. *Not*I digestion produced six fragments, N1 to N6 (Fig. 1B, lane 3), with sizes of 544, 444, 354, 116, 109, and 50 kbp, respectively, by PFGE. The sums of the fragment sizes were 1,617 kbp for both *Sfi*I and *Not*I digestions; this exact agreement, while convenient, is not meaningful.

Restriction map. The order of restriction fragments was determined by *Sfi*I-*Not*I (Fig. 1C) or *Not*I-*Sfi*I (Fig. 1D) double digestion and two-dimensional (2D) PFGE. After digestion with one enzyme and electrophoresis in one dimension, the DNA in a 2-mm-wide agarose slice the length and thickness of the original gel was then completely digested with the second enzyme and labeled within the agarose. The slice was embedded in a second, slightly thicker gel, and electrophoresis was conducted at right angles to the original direction. Ten (DD1 to DD10) double-digestion fragments were found. Eight of them were visible with ethidium bromide after PFGE; the smallest two were detected by autoradiography (not shown). Fragments S1 and S2 were found to contain three *Not*I sites each, and fragments S3 and S4 were found to contain no *Not*I sites. Fragments N2 and N5 each contained two *Sfi*I sites, and fragments N1, N3, N4, and N6 contained no *Sfi*I sites. The sizes of the various restriction fragments are given in Table 1.

S3 (DD3) was found in N2 but not in any other *Not*I fragments and therefore is located entirely within N2. N2 contained additional segments common to S1 and S2. Therefore, the order of these *Sfi*I fragments must be S1-S3-S2 or the reverse. S4 (DD8) was found only in N5, and N5 contained additional segments common to S1 and S2. Therefore, the order of fragments S1, S2, and S4 must be S1-S4-S2 or the reverse. These two orders are possible only if the map is circular.

Similarly, N1 (DD1) and N6 (DD7) are common only to S2. S2 also contained segments common to N2 and N5, so the order must be N2-(N1, N6)-N5 or the reverse, where the order of N1 and N6 is not determined. N3 (DD2) and N4 (DD5) are common only to S1. S1 also contained segments common to N2 and N5, so the order must be N2-(N3, N4)-N5 or the reverse. Again, these results are possible only if the map is circular.

To determine the correct order for the pairs N1-N6 and N3-N4, DNA fragments (19 bands [N1 to N6 and PN1 to PN13]) resulting from partial *Not*I cleavage (PN) with 1 U of *Not*I for 1 to 2 h were separated by electrophoresis in one dimension and then completely digested with *Not*I and ^{32}P labeled within the agarose. The completely digested fragments were then separated by electrophoresis conducted at right angles to the original direction (data not shown). PN1, the largest fragment (1,550 kbp), resolved into N1, N2, N3, N4, N5, and N6 (combined size of 1,617 kbp). PN13, the smallest partial cleavage fragment (163 kbp), resolved into N5 (109 kbp) and

* Corresponding author. Mailing address: Department of Medical Biochemistry and Genetics, 440 Reynolds Medical Building, Texas A&M College of Medicine, College Station, TX 77843-1114. Phone: (409) 845-2726. Fax: (409) 847-9481.

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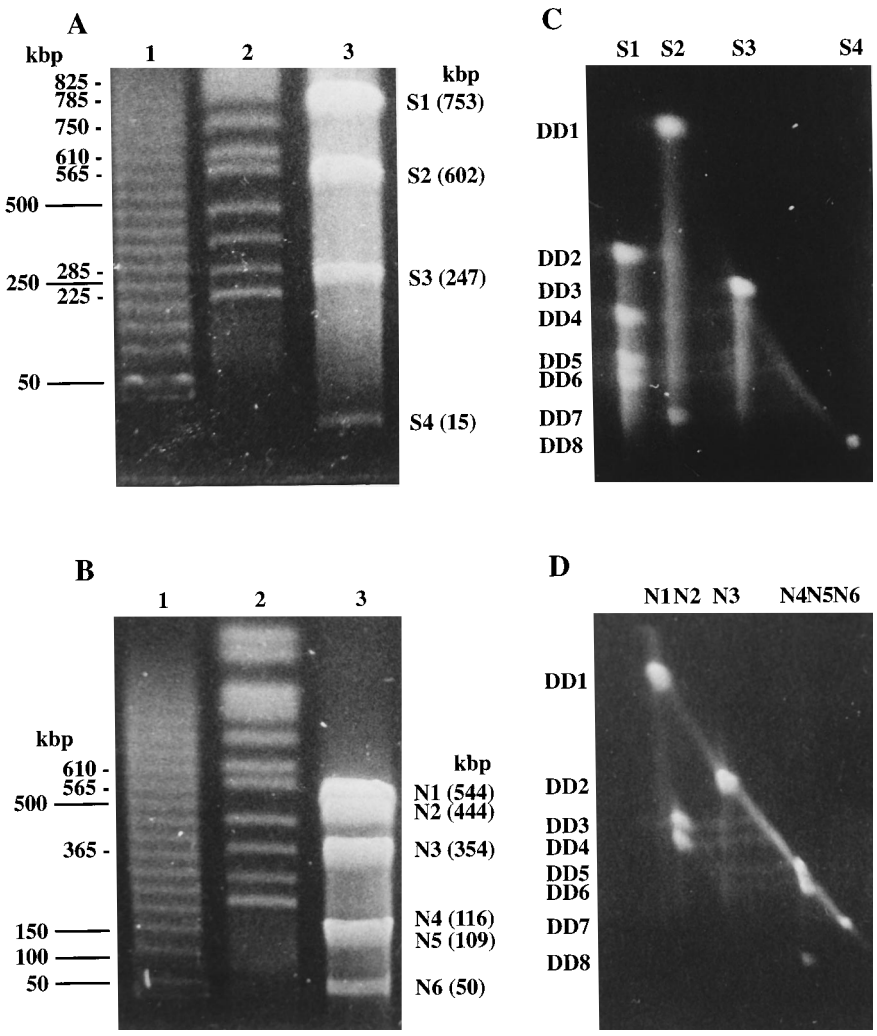

FIG. 1. Restriction fragments of *B. bacilliformis* genomic DNA separated by PFGE. (A) *Sfi*I. Lane 1, 50-kbp ladder of λ *c*I857 *S*am7 concatemers ranging from 48.5 to 970 kbp (Bio-Rad, Richmond, Calif.); lane 2, the 16 *Saccharomyces cerevisiae* chromosomes as size standards, ranging from 225 to 2,200 kbp (Bio-Rad, Richmond, Calif.); lane 3, *Sfi*I restriction fragments (S1 to S4) with sizes indicated. (B) *Not*I. Lane 1, 50-kbp ladder as in lane 1 of panel A; lane 2, *S. cerevisiae* chromosomes as in lane 2 of panel A; lane 3, *Not*I restriction fragments (N1 to N6) with sizes indicated. N4 and N5 were not resolved in this separation but were resolved by autoradiography. (C) 2D PFGE of *Sfi*I fragments (S1 to S4) separated in the horizontal direction, cleaved by *Not*I, and then separated in the vertical direction. DD9 and DD10 are not seen on this 2D gel but are seen by 2D PFGE under different electrophoretic conditions with visualization by autoradiography. (D) 2D PFGE of *Not*I fragments (N1 to N6) separated in the horizontal direction, cleaved by *Sfi*I, and then separated in the vertical direction. As in panel C, DD9 and DD10 must be visualized under different conditions.

N6 (50 kbp), so N5 and N6 are adjacent. The second-smallest fragment, PN12 (250 kbp), resolved into N5 (109 kbp) and N4 (116 kbp), so N5 and N4 are adjacent.

Confirming the order N4-N5-N6, PN11 (285 kbp) resolved into N4, N5, and N6. The next-largest partial cleavage fragment, PN10, was 664 kbp long, so no fragments were found at sizes consistent with the alternative arrangements of neighbors, N5-N3 (predicted size, 463 kbp) and N2-N6 (predicted size, 494 kbp).

The order of the four *Sfi*I fragments in the *B. bacilliformis* genome is S1-S3-S2-S4-S1. The order of the 6 *Not*I fragments is N1-N2-N3-N4-N5-N6-N1. That the genomic order of *Not*I digestion fragments corresponds to the order of their sizes is presumably accidental. The restriction map is diagrammed in Fig. 2.

Locations of rRNA genes and *Ceu*I sites. By hybridization, the *B. bacilliformis* rRNA genes were localized to DD4 and DD8. The 16S rRNA oligonucleotides used were from the 5′ region (nucleotides 1 to 14 and 4 to 28) and the middle region (nucleotides 726 to 750 and 727 to 746) of the *B. bacilliformis* 16S rRNA gene (GenBank accession number X60042) (3, 5, 12). The 5S rRNA oligonucleotide consisted of nucleotides 1 to 19 of the *B. bacilliformis* 5S rRNA gene (GenBank accession number L08503) (10).

*Ceu*I cuts within a 26-bp DNA recognition sequence which is found in a highly conserved region of bacterial 23S rRNA genes and which has not been found elsewhere in bacterial DNA (8). *Ceu*I would be expected to cleave the *B. bacilliformis* 23S rRNA gene (GenBank accession number L39095) once within positions 1836 to 1861.

Cleavage with *Ceu*I alone yielded two fragments. Cleavage with *Ceu*I and then with *Sfi*I resulted in the disappearance of fragments S1 and S4 (DD8), so at least one *Ceu*I site must be located in each of these fragments. *Ceu*I digestion followed by *Not*I digestion resulted in the disappearance of N2 and N5. The DNA segment common to S1 and N2 is DD4 (194 kbp)

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TABLE 1. DNA fragments generated by single or double digestion of the *B. bacilliformis* genome with *Sfi*I and/or *Not*I

Fragment size (kbp)	*Sfi*I fragment	*Not*I fragment	Double-digestion fragment(s)	Gene(s)
753	S1		DD2, DD4, DD5, DD6[a]	
602	S2		DD1, DD7, DD9, DD10[a]	
544		N1	DD1	
444		N2	DD3, DD4, DD10[a]	
354		N3	DD2	
247	S3		DD3	
194			DD4	*rrn*, *ial*
116		N4	DD5	
109		N5	DD6, DD8, DD9[a]	
89			DD6	
50		N6	DD7	*fla*
15	S4		DD8	*rrn*
5			DD9	
3			DD10	

[a] The multiple double-digestion fragments are components of the single-digestion fragment indicated in the same row.

and the DNA segment common to S4 and N5 is DD8 (15 kbp), so the location of the *Ceu*I sites is in agreement with the hybridization results.

The new DNA fragments with lengths of 644 and 89 kbp found by *Ceu*I-*Sfi*I digestion must be derived from S1 (753 kbp), indicating that the *Ceu*I site in S1 (and DD4) is about 89 (89 to 109) kbp from the *Sfi*I site that marks the boundary between S3 and S1. The data from the *Ceu*I-*Not*I digestion indicate that the *Ceu*I cleavage site is located about 105 to 142 kbp from the same *Sfi*I site. In the *Ceu*I-*Not*I digestion, N2 (444 kbp) and N5 (109 kbp) are replaced by new DNA fragments of 392 kbp, 89 kbp (which because of its width and brightness appears to be a double band), and 12 kbp in length. On the basis of the 392-kbp value, the *Ceu*I site would be located about 142 kbp from the *Sfi*I site, and on the basis of the 89-kbp value, the *Ceu*I site would be 105 kbp from the *Sfi*I site.

The 12-kbp fragment must be derived from N5 (109 kbp), indicating that this *Ceu*I site lies about in the middle of DD8. Locating the *Ceu*I site near the middle of DD8 is consistent with the results of a *Ceu*I-*Sfi*I digestion run on a standard gel in which new fragments with lengths of 8 and 7 kbp were found. These fragments must be derived from S4 (15 kbp), also indicating that the *Ceu*I site is near the center of S4 (DD8).

Location of invasion-associated locus and flagellin genes. By hybridization, the *B. bacilliformis* invasion-associated locus genes studied by Mitchell and Minnick (11) were localized to DD4. Invasion-associated locus oligonucleotides corresponded to sequences from positions 202 to 181 (on the negative strand of *ialA*) and positions 463 to 484 (on the positive strand of *ialB*) (GenBank accession number L25276) (11). ^{32}P-labeled oligonucleotide probes were prepared with T4 polynucleotide ki-

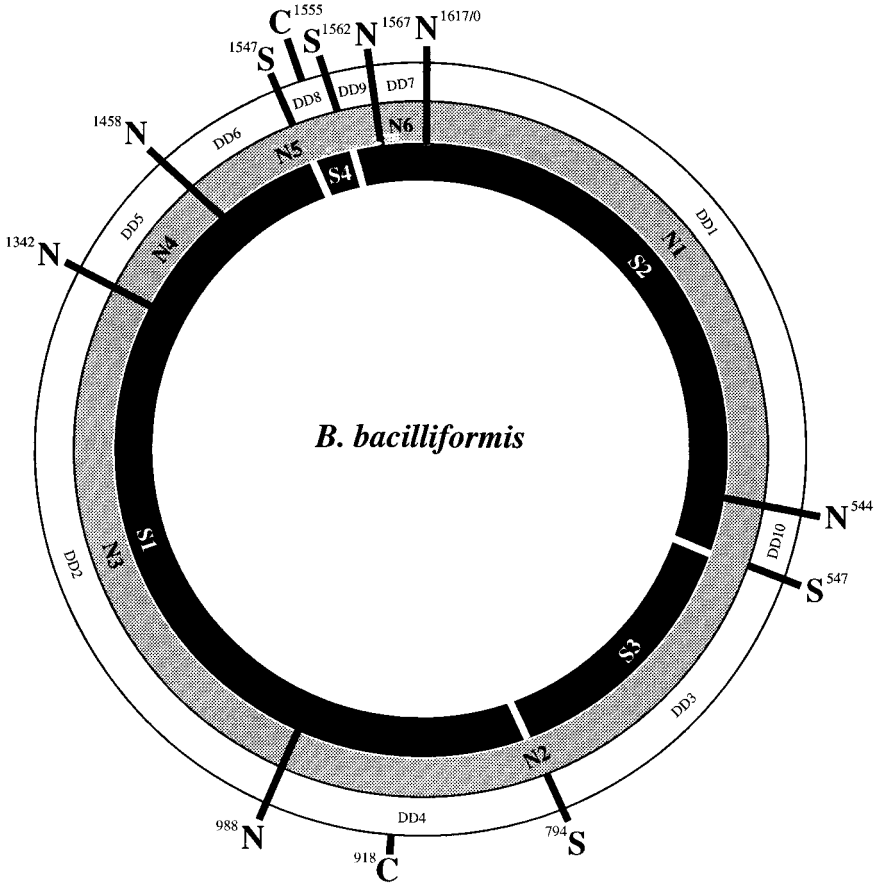

FIG. 2. Physical map of the *B. bacilliformis* genome. Locations (with positions in kilobase pairs) of two *Ceu*I (C), four *Sfi*I (S), and six *Not*I (N) sites are shown on a circular *B. bacilliformis* DNA molecule of 1,617 kbp. The locations of genomic DNA fragments DD1 to DD10, N1 to N6, and S1 to S4 are indicated in the inner circles.

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nase (GIBCO BRL, Gaithersburg, Md.) and [γ-^{32}P]ATP (Du Pont NEN, Boston, Mass.).

The *B. bacilliformis* flagellin gene cloned and sequenced by our laboratory (GenBank accession number L20677) was localized to DD7. The flagellin gene was available on a plasmid clone (1), which was linearized and labeled with ^{32}P with a random priming Ready-To-Go DNA labeling kit (Pharmacia, Piscataway, N.J.) according to the manufacturer's instructions.

For hybridization, nonradioactive DNA fragments or ^{32}P-, end-labeled fragments resulting from *Not*I-*Sfi*I digestion of the *B. bacilliformis* genome were separated in alternate lanes of pulsed-field gels. The gels were then stained with ethidium bromide and photographed. The DNA was nicked and vacuum transferred to a nylon membrane. The resulting blots were cut into strips each containing two lanes, one lane containing ^{32}P-, end-labeled reference fragments and the other lane containing the corresponding nonradioactive fragments to be probed. The individual strips were then hybridized to various probes and analyzed by autoradiography. DNA which hybridized to a probe could be unambiguously assigned to 1 of the 10 double-digestion fragments.

Genome size estimation. Time course reassociation of denatured *B. bacilliformis* genomic DNA was previously used to estimate the size of the *B. bacilliformis* genome to be one-sixth of that of *Escherichia coli*, or about 752 kbp (4). That *B. bacilliformis* would have a genome smaller than those of some *Mycoplasma* spp. while retaining genetically complex features such as flagella and a gram-negative cell wall was surprising.

PFGE permits a more accurate and direct physical measurement of the size of the genome than reassociation kinetics does. The sum of restriction fragment lengths showed that the size of the *B. bacilliformis* genome is twice that determined by time course reassociation techniques; the PFGE-based measurement seems to be in better agreement with the biological complexity of *B. bacilliformis*. The order of restriction fragments showed that they must have been cut from a circular DNA molecule. Additional evidence that the *B. bacilliformis* genome is circular is provided by the observations that cutting at the two *Ceu*I sites produced only two fragments and that uncleaved *B. bacilliformis* DNA did not enter the gel, but DNA incubated with S1 nuclease, which is believed to introduce only one break in circular DNA (1a, 2), produced a single, discrete DNA species (presumably a linearized form of the entire intact genome), migrating with an apparent size of 1,550 kbp. Also, the largest *Not*I partial cleavage fragment, PN1, which contains all six *Not*I complete cleavage fragments, entered the gel. PN1 must be the entire genome in linear form, resulting from a single *Not*I cleavage in the circular DNA. Both PN1 and S1 nuclease-treated genomic DNA, linearized but apparently complete DNA molecules, migrated with an apparent size of 1,550 kbp. The sum of the sizes of restriction fragments, which might contain multiple systematic errors, indicated that the size of the intact *B. bacilliformis* chromosome is 1,617 kbp for both *Not*I and *Sfi*I. We take the current best estimate for the genome size to be about 1,600 kbp.

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