

## Virulence Potential of *Ehrlichia chaffeensis* Strains of Distinct Genome Sequences<sup>∇†</sup>

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**Human monocytic ehrlichiosis, one of the most frequent life-threatening tick-borne zoonoses, is caused by *Ehrlichia chaffeensis* that lacks endotoxin and peptidoglycan. While sequence polymorphisms in several genes in *E. chaffeensis* strains have been reported, global genomic divergence and biological differences among strains are unknown. The objectives of the present study were to compare the genome sequences of strains of *E. chaffeensis* and to examine the virulence potentials of the strains with defined genome sequences. Genomic DNA was extracted from purified *E. chaffeensis* strains Wakulla and Liberty, and comparative genome hybridization was performed using a densely tiled microarray of 147,027 chromosome positions of the *E. chaffeensis* strain Arkansas genome. The results revealed that 4,663 and 5,325 positions in the chromosomes of strains Wakulla and Liberty, respectively, were different from those in the chromosome of strain Arkansas, including three common major polymorphic chromosomal regions. Of various functional categories, the differences were most concentrated in genes predicted to encode cell envelope proteins. Of all the open reading frames (ORFs), 21 *omp-1* (p28 gene) paralogs, nine genes encoding hypothetical proteins, two genes encoding ankyrin repeat proteins, and *hemE* contained the most differences. Several highly polymorphic ORFs were confirmed by sequencing. When the *E. chaffeensis* strains were inoculated into severe combined immunodeficiency mice, the order of the severity of clinical signs and the bacterial burden detected in mice was Wakulla > Liberty > Arkansas. Severe diffuse inflammation and granulomatous inflammation were evident in the livers of mice infected with strains Wakulla and Arkansas, respectively, but not in the livers of mice infected with strain Liberty. These results revealed distinct virulence phenotypes of *E. chaffeensis* strains with defined genome sequences.**

Human monocytotropic ehrlichiosis (HME) is an emerging tick-borne zoonosis that was discovered in the United States in 1986 (22). HME is caused by infection with *Ehrlichia chaffeensis*, an obligatory intracellular gram-negative bacterium in the order *Rickettsiales* that was isolated in 1990 (7). By 1997, 47 states had reported a total of 742 probable or confirmed cases of HME to the Centers for Disease Control and Prevention (25). The average reported annual incidence of HME during 2001 and 2002 was 0.6 case per million people (8). Several prospective epidemiologic studies have documented an increasing incidence of human ehrlichiosis (4, 13, 30). HME also has been reported in other parts of the globe, including Europe, Mexico, Argentina, Mali, Israel, and Thailand. *E. chaffeensis* has been identified most commonly in the Lone Star tick (*Amblyomma americanum*) (2), and white-tailed deer is considered to be the major reservoir in the United States (10).

HME generally is characterized by fever, headache, myalgia, anorexia, and chills and frequently is accompanied by leukopenia, thrombocytopenia, anemia, and elevated levels of serum hepatic aminotransferases (32). The manifestations of HME are broad, however, ranging from asymptomatic infection to death (32). Especially in patients with AIDS or other immu-

nosuppressive conditions, *E. chaffeensis* can cause overwhelming infection (23, 33, 39). The clinical signs are not induced by endotoxin, since *E. chaffeensis*, like other sequenced members of the family *Anaplasmataceae*, lacks genes for biosynthesis of lipid A and lipopolysaccharide and thus is incapable of producing endotoxin (17, 20).

Genetic diversity among *E. chaffeensis* human isolates initially was found in the sequence of the p28 (p28-19) gene encoding the 28-kDa major outer membrane protein (49). Further comparison of the p28-19 gene in other strains of *E. chaffeensis* led to proposal of three distinct groups of *E. chaffeensis* isolates (21). Cheng et al. (5) placed the *E. chaffeensis* isolates into three distinct genetic groups, groups I, II, and III, on the basis of restriction enzyme cleavage analysis and sequence data for the partial 28-kDa outer membrane protein multigene locus. *E. chaffeensis* isolates also differ in their numbers of repeat sequences within the p120 gene and the variable-length PCR target gene (33, 45). For example, the Wakulla strain has a six-repeat version of the variable-length PCR target gene (45), whereas the Arkansas and Liberty strains have four repeats. Although the genome of *E. chaffeensis* strain Arkansas has been sequenced (17), the global genomic divergence among *E. chaffeensis* strains has not been determined.

Pathogenic differences between strains have not been noted in patients with HME, since the present diagnostic methods cannot distinguish between strains. Furthermore, clinical outcomes are influenced by patient factors (age, underlying illness, and immunosuppressive conditions) and therapeutic in-

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terventions. As only a single strain (Arkansas) has been used so far for experimental infection of animals, there are no experimental data on biological or pathogenetic differences among *E. chaffeensis* strains. We thought that biological diversity among *E. chaffeensis* strains might be demonstrated clearly if the pathogenesis of *E. chaffeensis* strains with distinct genomic sequences could be compared under controlled conditions in an experimental animal model.

Immunocompetent mice clear *E. chaffeensis* Arkansas infection within 2 weeks and do not develop clinical signs (47). Consequently, severe combined immunodeficiency (SCID) mice have been used for studies of *E. chaffeensis* infection and pathogenesis. The SCID mouse model recapitulates several features of clinical signs and histopathology of the human disease (HME), including wasting, tissue and cell tropism, extensive tissue inflammation, splenomegaly, lymphadenopathy, and liver granulomas and necroses (47). Hence, we used SCID mice to compare the virulence potentials of the three *E. chaffeensis* human isolates with defined genome sequences. The phenotype and the genotype data for a given strain were obtained from bacteria having the same passage history. The results provide novel insights into the pathogenesis of *E. chaffeensis* strains and suggest new investigation targets for potential virulence factors.

#### MATERIALS AND METHODS

**Bacteria and cell lines.** *E. chaffeensis* strains Arkansas (from a patient residing in Arkansas) and Wakulla and Liberty (from patients residing in Florida) were kindly provided by C. Paddock and W. L. Nicholson at the Centers for Disease Control and Prevention, Atlanta, GA. *E. chaffeensis* strains were propagated in DH82 cells as previously described (38). Infected cells were lysed by nitrogen cavitation, and liberated bacteria were purified by Percoll density gradient centrifugation as previously described (27, 28).

**DNA tiling microarray and sequencing.** Genomic DNA was extracted from the purified organisms using a QIAamp DNA blood mini kit (QIAGEN, Valencia, CA). The contamination level of host cell DNA in the purified bacterial DNA preparation was less than 1% as determined by PCR using host-specific glyceraldehyde-3-phosphate dehydrogenase primers as previously described (17). The high-density *E. chaffeensis* DNA microarray was constructed at NimbleGen Systems (Madison, WI). Cy3- and Cy5-labeled probes were synthesized from genomic DNA (1 µg) of Arkansas, Wakulla, and Liberty using a randomly primed reaction as previously described (1). Labeled genomic DNA was hybridized to arrays in 1× NimbleGen hybridization buffer (NimbleGen); the arrays were scanned at a 5-µm resolution using a Genepix 4000b scanner (Axon Instruments, Union City, CA); and pixel intensities were extracted using NimbleScan software (NimbleGen). The genomic loci concerned were amplified by PCR with several primer sets designed based on conserved sequences for each locus. After purification with a QIAquick gel extraction kit (QIAGEN), the PCR products were used for direct sequencing.

**Infection of mice.** Three- to 4-week-old male ICR-*scid* mice were obtained from Taconic (Tarrytown, NY) and infected intraperitoneally with 10<sup>6</sup> *E. chaffeensis*-infected DH82 cells (typically >95% infected) containing approximately the same number of bacteria. The number of bacteria was estimated as previously described (48). Determination of the viability of organisms in infected DH82 cells was performed with a LIVE/DEAD BacLight bacterial viability kit (Molecular Probes, Eugene, OR). Infected DH82 cells were harvested, washed, and resuspended in phosphate-buffered saline (137 mM NaCl, 2.68 mM KCl, 1.47 mM KH<sub>2</sub>PO<sub>4</sub>, 8.06 mM Na<sub>2</sub>HPO<sub>4</sub>), and 0.5 ml was injected into the peritoneal cavity with a 26-gauge needle. Heparinized blood specimens were collected from the submandibular sinus of mice anesthetized by inhalation with CO<sub>2</sub>. The experiment was terminated at day 15 postinfection (PI) according to the Institutional Animal Care and Use Committee regulations, since mice inoculated with the Wakulla strain became moribund by this time. The tissues were harvested and fixed in Histochoice (Amresco, Solon, OH) for histopathologic examination or were stored in RNAlater (QIAGEN) for PCR analyses. The levels of total serum albumin and serum aminotransferases (aspartate and ala-

nine aminotransferase) were measured with a 911 automatic analyzer (Hitachi, Tokyo, Japan) at 15 days PI.

**Real-time PCR and reverse transcriptase PCR.** DNA was extracted from blood, spleen, and liver specimens with a QIAamp blood kit (QIAGEN). To determine the numbers of *E. chaffeensis* bacteria, a real-time PCR assay was performed in triplicate to amplify the *E. chaffeensis* 16S rRNA gene as previously described (6). Total RNA was extracted from the spleen and liver using an RNeasy kit (QIAGEN). Total cellular RNA (2 µg) was treated with DNase I (Invitrogen, Carlsbad, CA) and was reverse transcribed with SuperScript III reverse transcriptase (Invitrogen), and the cDNA was used in the PCR as previously described (6).

**Statistical analysis.** The unpaired two-tailed *t* test was performed to test the significance of differences between different groups. A *P* value of <0.05 was considered significant.

#### RESULTS

**CGH.** Based on the group designation of Cheng et al. (5) and the availability of strains, we selected the genomic sequences of the Wakulla (group II) and Liberty (group III) strains of *E. chaffeensis* for comparison with the genomic sequence of the Arkansas strain (group I). For comparative genome hybridization (CGH) analysis, a high-density DNA microarray was constructed by selecting 29-mer oligonucleotides every eight bases in the genome sequence of *E. chaffeensis* Arkansas (GenBank accession no. CP000236), resulting in a total of 147,027 chromosome positions on each strand (GEO accession no. GPL4360). With this approach, each nucleotide in the genome was covered with a total of six or eight overlapping oligonucleotides in the array (Fig. 1A [oligonucleotides for only one DNA strand are shown]). We purified bacteria from infected host cells as previously described (17) so that we could isolate *E. chaffeensis* genomic DNA that was almost free of host DNA. Arkansas genomic DNA was labeled with Cy3. Wakulla and Liberty DNA probes were labeled with Cy5, and each of the probes was hybridized with the Arkansas oligonucleotides on the microarray. Cy3/Cy5 ratios for each pair (Arkansas/Wakulla and Arkansas/Liberty) were determined across the entire 147,027 chromosome positions on both DNA strands, and the means of the two strands at each position were plotted (Fig. 1A) (GEO accession no. GSM137280 and GSM137281). Overall, the gene content was highly conserved (>99%) compared to the conservation observed in similar studies done with *Helicobacter pylori* (40) and *Staphylococcus aureus* (14). The median plus two times the standard deviation of the Cy3/Cy5 ratios for each pair of all chromosome positions was used as a threshold to identify differences at each nucleotide position in the *E. chaffeensis* sequence in the array. The threshold was 2.78 for Arkansas/Wakulla and 3.29 for Arkansas/Liberty. Wakulla differed from Arkansas at 4,663 positions in the genome (3.2%), and Liberty differed from Arkansas at 5,325 positions (3.6%) (Fig. 1B). These differences were distributed in 361 open reading frames (ORFs) and 133 intergenic regions of Wakulla and in 357 ORFs and 200 intergenic regions of Liberty. The numbers of differences in various functional categories divided by the total length of the ORFs in the categories are shown in Table 1. In the genomes of both Wakulla and Liberty, genes encoding cell envelope proteins (total length, ~46 kb) were most different from the genes in the Arkansas strain, whereas genes for housekeeping and metabolic functions had fewer differences. In particular, a 30-kb contiguous region containing 22 OMP-1 (p28) gene paralogs,

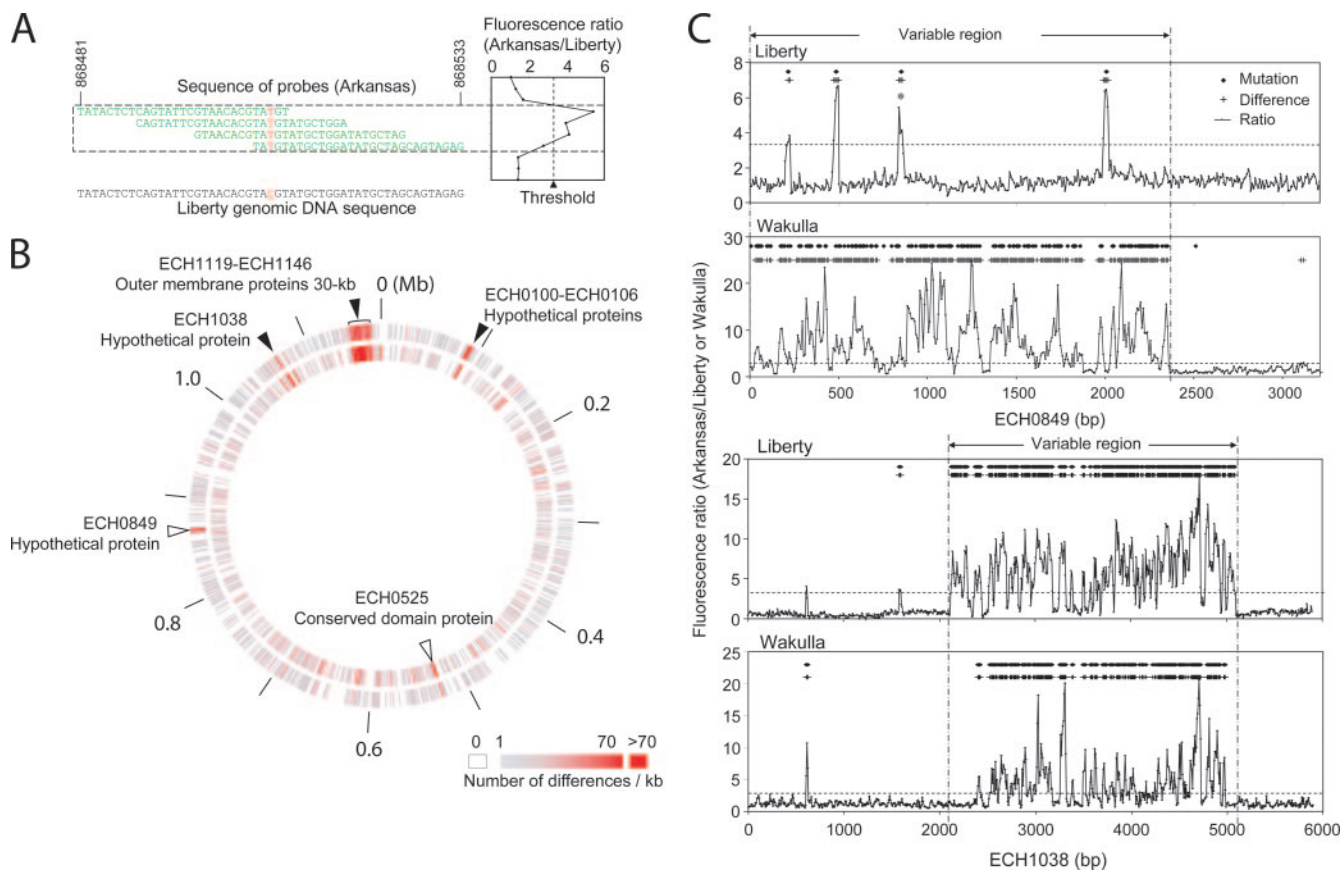


FIG. 1. CGH of *E. chaffeensis* strains. (A) Multiple overlapping probes cover each nucleotide. A mutation at a site, indicated by red, perturbs hybridization of the sample to all probes. The representative example is a mutation in Liberty ECH\_0849, indicated by an asterisk in panel C. The sequence of probes on one of the strands is represented. (B) *E. chaffeensis* strains Wakulla and Liberty were compared with the Arkansas strain using an array containing a total of 294,050 29-mer probes spaced, on average, every 8 bp throughout the genome of *E. chaffeensis* Arkansas. Inner circle, Arkansas versus Liberty; outer circle, Arkansas versus Wakulla. The open and solid arrowheads indicate remarkably different regions. (C) ECH\_0849 and ECH\_1038: CGH results revealed different positions and actual mutations. Sequencing was used to validate the sensitivity and specificity of CGH. The horizontal dotted line indicates the threshold of the Cy3/Cy5 ratio used to indicate a difference. The diamonds and plus signs indicate positions of mutated bases and probes with differences, respectively. The variable regions of ECH\_0849 and ECH\_1038 in the Wakulla and Liberty strains compared with strain Arkansas are between two vertical dashed and dotted lines.

including the six OMP-1 (p28) gene paralogs reported previously by Cheng et al. (5), had many mutations when Wakulla and Arkansas were compared and when Liberty and Arkansas were compared (Fig. 1B and Table 1). Of all the ORFs, 21 *omp-1* (p28 gene) paralogs, nine genes encoding hypothetical proteins, two genes encoding ankyrin repeat proteins, and *hemE* contained the most differences (Table 2).

The following three major genomic regions were consistently different when Arkansas was compared with Wakulla or Liberty: the 30-kb region from ECH\_1119 to ECH\_1146 encoding 22 OMP-1 paralogs mentioned above; ECH\_1038 (GenBank accession no. YP\_507823), which encodes a hypothetical 222,638-Da protein; and the region from ECH\_0100 to ECH\_0106 encoding hypothetical proteins (Fig. 1B and Table 2). Wakulla had a deletion of ECH\_0100 to ECH\_0104. The ECH\_0849 ORFs (GenBank accession no. YP\_507645), which encode hypothetical 121,401-Da proteins, differed at many positions in Wakulla and Arkansas but were similar in Liberty and Arkansas (Fig. 1C). Both ECH\_1038 and ECH\_0849 were transcribed by the Wakulla and Liberty strains in DH82 cell

cultures and in the spleens of SCID mice (data not shown), implying that these ORFs are functional genes.

To confirm the CGH data, the ECH\_0849, ECH\_1038, and *hemE* genes of Wakulla and Liberty were sequenced (GenBank accession no. DQ888319, DQ912824, DQ915166, DQ915979, DQ924562, and DQ924563). The results revealed by CGH for different chromosome positions and actual mutations detected by sequencing are similar for ECH\_0849 and ECH\_1038 of strains Wakulla and Liberty (Fig. 1C). A comparison of the sequences indicated that the sensitivities of probes on the array for detection of differences between strains Wakulla and Arkansas and between strains Liberty and Arkansas were 81.0 and 87.8%, respectively (see Table S1 in the supplemental material). The specificities of probes on the array for detection of differences between strains Arkansas and Wakulla and between strains Arkansas and Liberty were 98.0 and 99.4%, respectively. The sensitivity and specificity of probes for strains Wakulla and Liberty combined were 83.6 and 98.7%, respectively (see Table S1 in the supplemental material). The net sensitivity of detection of mutations (per-

TABLE 1. Distribution of differences from strain Arkansas by functional category

Category	Total length (bp)	Difference from strain Arkansas in strain:				Avg rate (kb <sup>-1</sup> )
		Wakulla		Liberty		
		No.	Rate (kb <sup>-1</sup> )	No.	Rate (kb <sup>-1</sup> )	
Cell envelope	45,936	1,342	29.21	1,511	32.89	31.05
rRNA	4,382	89	20.31	0	0.00	10.16
Hypothetical proteins	134,832	1,132	8.40	1,031	7.65	8.02
Disrupted reading frame	171	0	0.00	2	11.70	5.85
Conserved hypothetical proteins	88,142	364	4.13	642	7.28	5.71
Intergenic regions	230,774	592	2.57	1,108	4.80	3.68
Unknown function	93,622	251	2.68	318	3.40	3.04
Central intermediary metabolism	1,698	3	1.77	4	2.36	2.06
Protein synthesis	91,797	165	1.80	195	2.12	1.96
Mobile and extrachromosomal element functions	4,763	9	1.89	9	1.89	1.89
Biosynthesis of cofactors, prosthetic groups, and carriers	51,912	119	2.29	53	1.02	1.66
tRNA	2,860	4	1.40	5	1.75	1.57
Transcription	28,137	58	2.06	22	0.78	1.42
Cellular processes	29,157	36	1.23	46	1.58	1.41
Energy metabolism	88,473	148	1.67	98	1.11	1.39
Transport and binding proteins	34,129	32	0.94	57	1.67	1.30
Regulatory functions	12,315	9	0.73	23	1.87	1.30
Fatty acid and phospholipid metabolism	15,012	23	1.53	15	1.00	1.27
Amino acid biosynthesis	24,183	31	1.28	29	1.20	1.24
Protein fate	96,477	131	1.36	82	0.85	1.10
DNA metabolism	61,412	83	1.35	52	0.85	1.10
Purines, pyrimidines, nucleosides, and nucleotides	38,767	50	1.29	31	0.80	1.04
Structural RNA	746	0	0.00	0	0.00	0.00

cent detection) in the sequences in Wakulla and Liberty combined was 97.3% (see Table S1 in the supplemental material). CGH and sequencing results revealed the presence of polymorphic and conserved regions within ECH\_0849 and ECH\_1038 (Fig. 1C).

ECH\_1038 is paralogous to ECH\_1037 and ECH\_1036 in the same orientation, and these three ORFs and their synteny are conserved among sequenced *Ehrlichia* species, including *Ehrlichia ruminantium* Welgevonden (Erum7970, Erum7960, Erum7950) and *Ehrlichia canis* Jake (Ecaj\_0838, Ecaj\_0835, Ecaj\_0834). The ECH\_0849 locus also is conserved among sequenced *Ehrlichia* species.

**Clinical signs and bacterial burden.** To compare the levels of pathogenesis of *E. chaffeensis* Liberty, Wakulla, and Arkansas, for each strain, five SCID mice were intraperitoneally inoculated with the same number of freshly harvested DH82 cells containing approximately the same number of viable bacteria. The numbers of Wakulla, Arkansas, and Liberty cells were  $55 \pm 7$ ,  $57 \pm 2$ , and  $68 \pm 6$  bacteria per DH82 cell, respectively. Retrospectively, regression analysis with Microsoft Excel showed that the correlation coefficient ( $r$ ) was 0.932 for the bacterial genome equivalent (as determined by the real-time PCR) versus the bacterial count (see Fig. S1 in the supplemental material). The viabilities of bacteria used for inoculation were >99% as determined with the LIVE/DEAD BacLight bacterial viability kit. Although this kit is prescribed for determining the viability of extracellular bacteria, we found that it also is useful for determination of the viability of obligatory intracellular bacteria; live bacteria were stained green with SYTO9, and dead bacteria were stained red with propidium iodide (an example of a cell containing both live and dead bacteria is shown in Fig. S2 in the supplemental material). The y-axis intercepts (day 0) of the fitted curves for the

Wakulla, Liberty, and Arkansas strains in blood were 2,430, 892, and 2,279 genome equivalents/ $\mu$ g DNA, respectively (Fig. 2B).

Mice inoculated with Wakulla and Liberty rapidly lost body weight starting on day 10 PI (Fig. 2A). In contrast, the body weight loss of mice inoculated with Arkansas occurred more slowly. Anorexia and wasting were evident with Wakulla-inoculated mice even earlier, as they stopped gaining body weight starting 4 days PI, while Liberty- and Arkansas-inoculated mice gained amounts of body weight similar to the amounts gained by mock-infected mice until day 10 PI (Fig. 2A).

As determined by real-time PCR, the numbers of Wakulla and Liberty bacteria in the blood increased exponentially (Fig. 2B). The numbers of Wakulla and Liberty bacteria in the blood increased about 90- and 60-fold, respectively, from day 5 to day 15 PI, whereas the number of Arkansas bacteria increased only 3-fold as determined by quantitative PCR (Fig. 2B). The modest increase in the bacterial load with the Arkansas strain in the present study was similar to the previous observation for C.B-17-*scid* mice inoculated with the Arkansas strain (47). On day 15 PI, the numbers of Wakulla and Liberty bacteria in the blood were approximately 140- and 40-fold greater than the number of Arkansas bacteria, respectively (Fig. 2B). The differences in bacterial loads between Liberty- and Wakulla-inoculated mice were more pronounced in the liver than in the spleen or blood. The livers of Wakulla- and Liberty-inoculated mice had approximately 900- and 10-fold more bacteria, respectively, than the livers of mice inoculated with Arkansas (Fig. 2C). The spleens of Wakulla- and Liberty-inoculated mice had 170- and 50-fold more bacteria, respectively, than the spleens of mice inoculated with Arkansas (Fig. 2C).

**Liver pathology and clinical pathology.** The liver is one organ where prominent pathological changes were demon-

TABLE 2. Most different ORFs in CGH analyses of *E. chaffeensis* strains

ORF	Gene	Direction	Length (bp)	Protein	No. of differences in CGH		
					Wakulla	Liberty	Total
ECH_1038		-	5,892	Hypothetical protein	201	281	482
ECH_0106		+	2,142	Hypothetical protein	176	184	360
ECH_0849		+	3,210	Hypothetical protein	234	13	247
ECH_0525		+	2,001	Conserved domain protein	98	135	233
ECH_0653		-	12,942	Ankyrin repeat protein	75	115	190
ECH_1127	<i>omp-1V</i>	+	840	Major outer membrane protein OMP-1V	78	95	173
ECH_1142	<i>omp-1F</i>	+	843	Major outer membrane protein OMP-1F	88	84	172
ECH_1126	<i>omp-1U</i>	+	888	Major outer membrane protein OMP-1U	64	104	168
ECH_1123	<i>omp-1Q</i>	+	894	Major outer membrane protein OMP-1Q	74	86	160
ECH_1133	<i>omp-1H</i>	+	897	Major outer membrane protein OMP-1H	71	86	157
ECH_1125	<i>omp-1T</i>	+	819	Major outer membrane protein OMP-1T	61	91	152
ECH_1129	<i>omp-1W</i>	+	852	Major outer membrane protein OMP-1W	58	93	151
ECH_1132	<i>omp-1S</i>	+	876	Major outer membrane protein OMP-1S	71	80	151
ECH_1131	<i>omp-1Y</i>	+	858	Major outer membrane protein OMP-1Y	75	75	150
ECH_1124	<i>omp-1P</i>	+	858	Major outer membrane protein OMP-1P	68	77	145
ECH_1135	<i>omp-1A</i>	+	882	Major outer membrane protein OMP-1A	45	95	140
ECH_1134	<i>omp-1Z</i>	+	903	Major outer membrane protein OMP-1Z	44	94	138
ECH_1143	<i>p28</i>	+	846	Major outer membrane protein P28	68	70	138
ECH_1130	<i>omp-1X</i>	+	828	Major outer membrane protein OMP-1X	57	79	136
ECH_1136	<i>omp-1B</i>	+	852	Major outer membrane protein OMP-1B	47	77	124
ECH_1137	<i>omp-1C</i>	+	843	Major outer membrane protein OMP-1C	84	39	123
ECH_1121	<i>omp-1N</i>	+	855	Major outer membrane protein Omp-1N	50	64	114
ECH_1128		+	591	Conserved hypothetical protein	38	70	108
ECH_0684		-	4,392	Ankyrin repeat protein	59	44	103
ECH_1035		-	548	Conserved hypothetical protein, Deg	33	61	94
ECH_1140	<i>omp-1E</i>	+	837	Major outer membrane protein OMP-1E	75	9	84
ECH_1144	<i>p28-1</i>	-	816	Major outer membrane protein P28-1	51	26	77
ECH_1119	<i>omp-1M</i>	+	897	Major outer membrane protein OMP-1M	28	30	58
ECH_1044		-	357	Conserved hypothetical protein	31	21	52
ECH_0150		-	2,019	Hypothetical protein	35	13	48
ECH_0282		+	675	Hypothetical protein	28	18	46
ECH_0030	<i>hemE</i>	-	1,005	Uroporphyrinogen decarboxylase	27	9	36
ECH_1139	<i>omp-1D</i>	+	861	Major outer membrane protein OMP-1D	23	13	36

strated in patients with HME, in SCID mice infected with *E. chaffeensis* Arkansas, and in immunocompetent mice infected with the HF strain (*Ixodes ovatus* ehrlichia isolated from the *I. ovatus* tick in Japan [42]) (29, 31, 43, 47). The HF strain is an ehrlichial isolate that is most closely related to *E. chaffeensis* (the 16S rRNA sequence identity with *E. chaffeensis* Arkansas was 98.21% when 1,449 bp aligned base sequences were compared [42]). Hence, we compared the liver histopathologies in the four groups of mice. Compared with the livers of mock-inoculated control mice, parenchymal cells were much smaller and the sinusoidal space was larger in the livers of mice infected with any of the three *E. chaffeensis* strains (Fig. 3A). The sinusoidal irregular dilation was most prominent with the Wakulla strain, distorting liver cell cord arrangements (Fig. 3A). Severe granulomatous inflammatory leukocyte infiltration in the liver was evident with Arkansas infection, disorganizing liver cell cords, but coagulation necrosis was not observed (Fig. 3A). This is similar to the previous observations made with C.B-17-*scid* mice inoculated with the Arkansas strain (47) (Fig. 3A). Wakulla infection induced profound and diffuse leukocyte infiltration, but a large granulomatous aggregation of leukocytes was rare (Fig. 3A). Apoptotic cells with small nuclei and atrophy and degeneration of parenchymal cell cords were evident in the livers of mice inoculated with Wakulla (Fig. 3A). In contrast, the livers of mice inoculated with Liberty were

almost free from leukocyte infiltration, granuloma, or degenerating parenchymal cells (Fig. 3A).

Since the cytoplasm of liver parenchymal cells was much smaller in three groups of infected mice, carbohydrate-specific periodic acid-Schiff (PAS) staining was performed to compare the glycogen amounts in the cytoplasm. PAS staining showed abundant granular glycogen accumulation in the cytoplasm of the parenchymal cells of the control mice. In contrast, glycogen was almost completely depleted in the parenchymal cells of mice infected with any of the three strains (Fig. 3B). The aspartate transaminase activities at day 15 PI were significantly elevated ( $575 \pm 29$  U/liter;  $n = 4$ ) in the sera from mice infected with Wakulla but not in the sera from mice infected with Arkansas or Liberty compared to the activities in the sera from the control mice ( $100$  to  $200 \pm$  U/liter; five mice in each group). The levels of serum aspartate transaminase in mice infected with Wakulla were similar to those reported for mice inoculated with the HF strain at days 7 and 9 PI just prior to death (43).

Anemia is one of clinical signs observed in human HME patients (32). Although anemia was not reported in any previous studies of SCID mice inoculated with the *E. chaffeensis* Arkansas strain, blood specimens taken from Wakulla-inoculated mice on day 15 PI had a lower hematocrit value (19.6% packed cell volume) and an increased

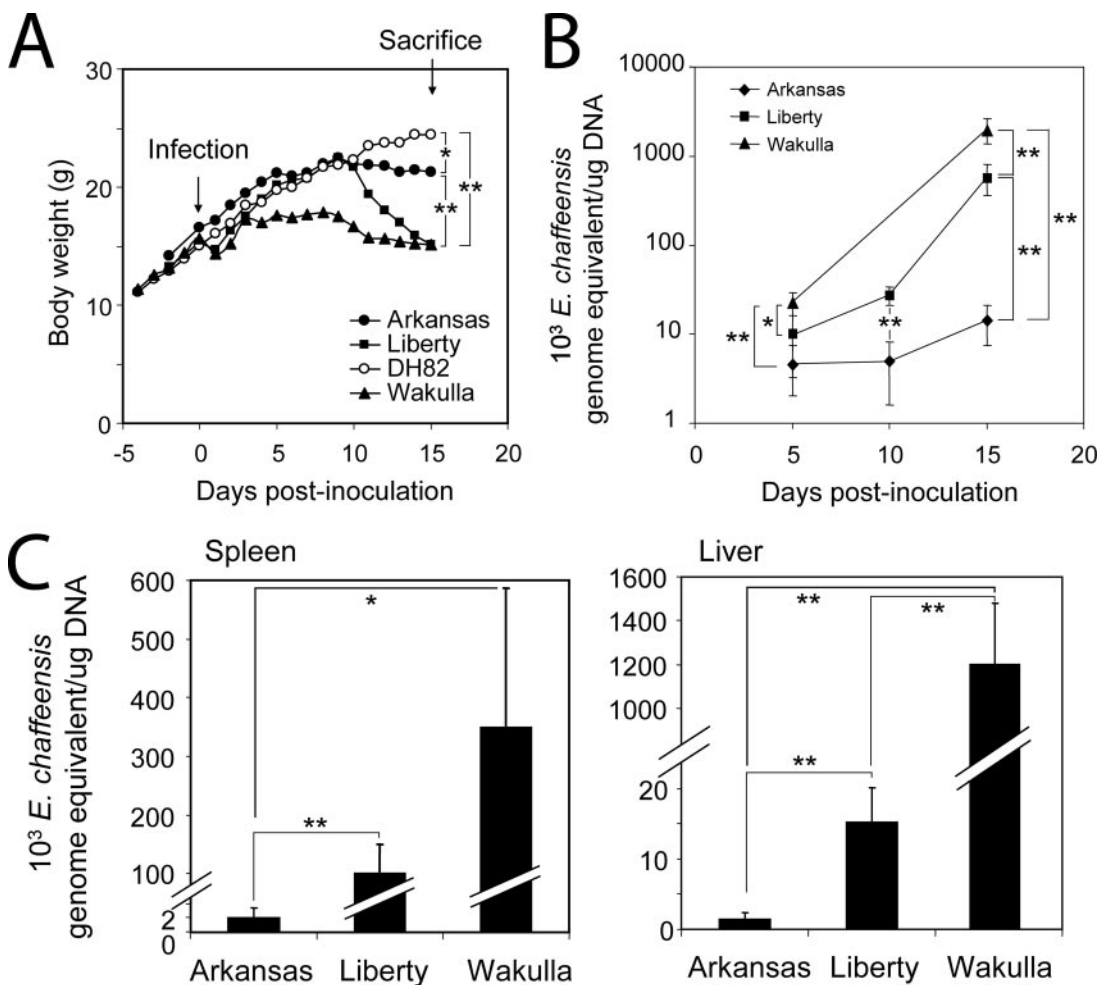


FIG. 2. *E. chaffeensis* strains differ in virulence. (A) Body weights of mice inoculated with DH82 cells infected with *E. chaffeensis* Arkansas, Liberty, or Wakulla or with uninfected DH82 cells (control). The values are means ( $n = 5$ ; on day 6 one mouse inoculated with Wakulla died and therefore the data for four mice in this group were used after day 7). One asterisk,  $P < 0.05$  at day 15 PI (as determined by an unpaired two-tailed  $t$  test); two asterisks,  $P < 0.01$  at day 15 PI (as determined by an unpaired two-tailed  $t$  test). (B) Bacterial loads in the blood of mice as determined by real-time PCR based on the 16S rRNA gene of *E. chaffeensis* ( $n = 5$ , except for mice inoculated with Wakulla after day 6 [ $n = 4$ ]). One asterisk,  $P < 0.05$ ; two asterisks,  $P < 0.01$  (as determined by an unpaired two-tailed  $t$  test). (C) Bacterial loads on day 15 PI in the spleens and livers of mice inoculated with *E. chaffeensis*, as determined by real-time PCR of the 16S rRNA gene of *E. chaffeensis* ( $n = 5$ , except for mice inoculated with Wakulla [ $n = 4$ ]). One asterisk,  $P < 0.05$ ; two asterisks,  $P < 0.01$  (as determined by an unpaired two-tailed  $t$  test).

number of reticulocytes, indicating severe anemia. In contrast, mice inoculated with the Arkansas or Liberty strain did not show significant anemia or reticulocytes in their blood. In addition, Wakulla-inoculated mice developed poor hemostasis, perhaps due to thrombocytopenia. One Wakulla-inoculated mouse died on day 6 PI, likely due to poor hemostasis after blood sample collection. Therefore, blood specimens could not be collected from the remaining Wakulla-inoculated mice until day 15 PI. Wakulla- and Arkansas-inoculated mice had six- to sevenfold-greater splenomegaly than mock-inoculated control mice, whereas Liberty-inoculated mice had two- to threefold-greater splenomegaly than mock-inoculated control mice (data not shown). These results indicated that the virulence and pathogenesis features of *E. chaffeensis* Wakulla and Liberty in SCID mice were distinct from each other and from those of Arkansas.

**DISCUSSION**

The present study demonstrated the utility of CGH using the densely tiled DNA microarray for comparison of *E. chaffeensis* strains. Like the genomic contents of other intracellular bacteria (3, 16, 17, 19), the genomic contents were found to be fairly conserved among *E. chaffeensis* strains. Thus, this type of microarray is more suitable than a gene content-based microarray for discerning diversity among *E. chaffeensis* strains. The high sensitivity and specificity demonstrated in the present study ensured the reliability of the data from CGH. Of the various functional categories, differences among the three *E. chaffeensis* strains were most concentrated in genes predicted to encode cell envelope proteins. Bacterial envelope proteins are at the host-bacterium interface and are involved in binding, internalization, signal transduction, nutrient transport, and immunoavoidance. Strain variation among envelope proteins of

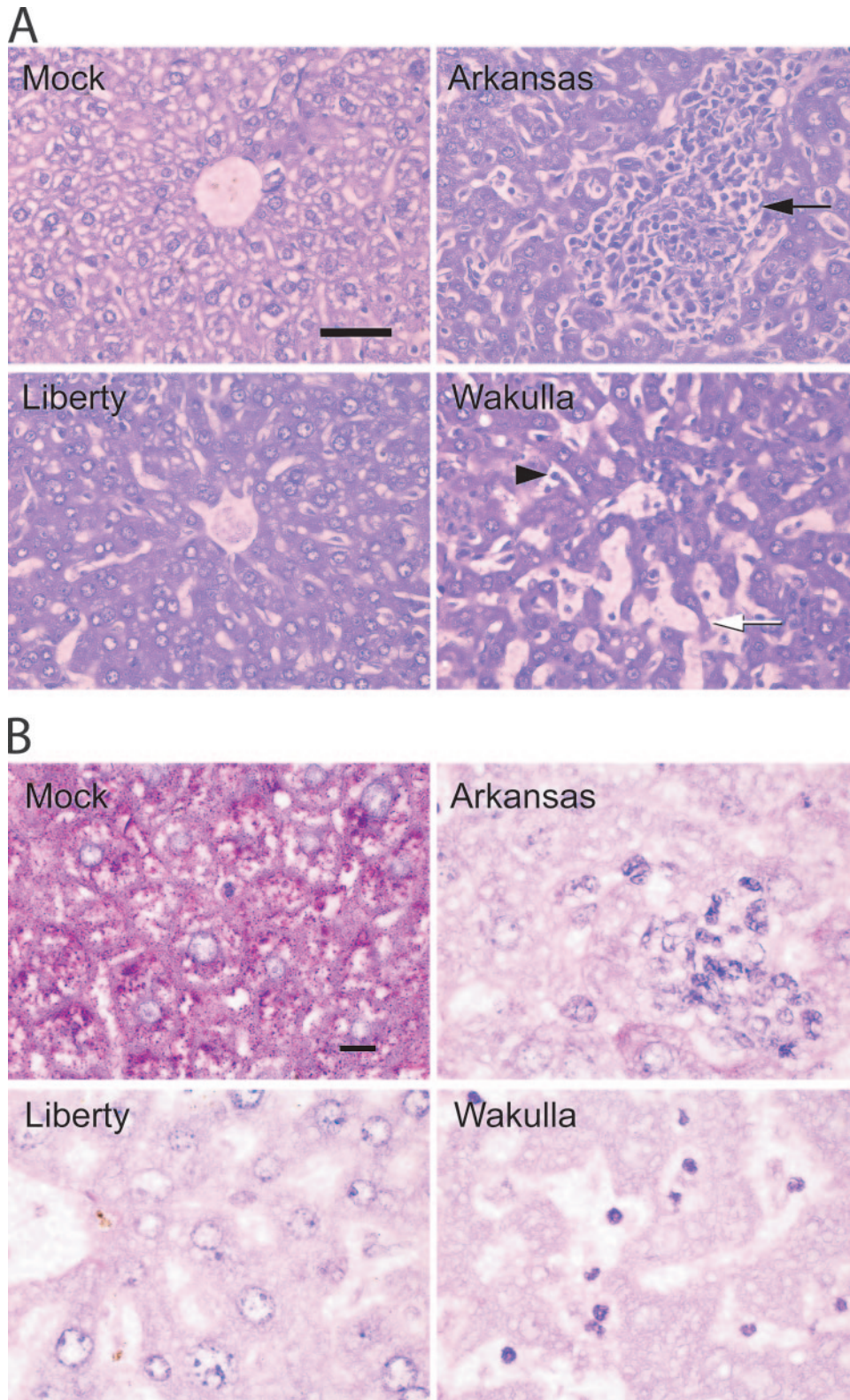


FIG. 3. (A) Liver histopathology of mice inoculated with *E. chaffeensis*. Note the diffuse inflammatory cell infiltration in the sinusoids (arrowhead) in the mouse infected with the Wakulla strain, the severe granulomatous inflammation (arrow) in the mouse infected with the Arkansas strain, and the absence of lesions in the mouse infected with the Liberty strain. Severe and irregular dilatation of sinusoids and atrophy and degeneration of liver cell cords (arrow) were evident in the mouse infected with the Wakulla strain. Hematoxylin and eosin stain was used. Bar = 50  $\mu$ m. (B) Liver glycogen depletion. The control mouse liver has massive glycogen accumulation, which is depleted in the mice infected with the three strains. PAS stain was used. Bar = 10  $\mu$ m.

*E. chaffeensis* is expected to influence some of these functions and, consequently, the ability of bacteria to infect and proliferate in the host cells and cause diseases. Therefore, comparative functional studies of the products of the polymorphic envelope genes may shed light on *E. chaffeensis* pathogenesis in the future. Other polymorphic genes in *E. chaffeensis* strains are genes encoding ankyrin repeat proteins. Genomes of *Ehrlichia* and *Anaplasma* species encode several ankyrin repeat proteins per genome (17), one of which has been reported to interact with host cell chromosomes (34) and a host cell phosphatase (18). Strain polymorphisms of ankyrin repeat proteins may alter the host cell transcription or signaling process and, therefore, the survival of these obligatory intracellular bacteria.

Several genes highly polymorphic in *E. chaffeensis* strains, such as ECH\_0100 to ECH\_0106, ECH\_0849, and ECH\_1038, encode hypothetical proteins that have not been studied previously. PSORTB analysis (<http://psort.ims.u-tokyo.ac.jp/>) showed that proteins encoded by ECH\_0849 and ECH\_1038 are predicted to be inner and outer membrane proteins, respectively. Our study showed that genes encoding these proteins were expressed in infected cell cultures, as well as in infected mice, and thus are functional rather than degenerating on the way to elimination. This finding may have a broader implication, since ECH\_0849 and ECH\_1038 homologs (also encoding hypothetical proteins) were found in the genomes of *E. canis* and *E. ruminantium*, which are responsible for canine ehrlichiosis and heartwater of ruminants (37), respectively, suggesting that the gene products have functions unique to the genus *Ehrlichia*. In the future, when larger numbers of *E. chaffeensis* strains are compared using this type of CGH, a more distinct *E. chaffeensis* genomic pattern is expected to emerge.

The Arkansas strain was isolated from a young man with relatively mild clinical signs, including fever, headache, pharyngitis, nausea, vomiting, and dehydration. Cervical lymphadenopathy, splenomegaly, mild thrombocytopenia, and leukopenia also were observed. The patient defervesced within 24 to 48 h after tetracycline treatment and quickly recovered from the illness (7). Unfortunately, information on clinical signs of the patients from whom Wakulla and Liberty strains were isolated (45) is not available. Thus, it is not feasible to relate the present findings to patients' clinical signs or outcomes. Nonetheless, the diverse clinical spectra observed in the SCID mice infected with three strains of *E. chaffeensis* in the present study are similar to those seen in patients with HME. The clinical spectrum of HME ranges from mild to life threatening, with a case fatality rate of 2 to 3%; approximately 60 to 70% of infected patients have been hospitalized (9, 11, 12, 32, 35).

One of the most common laboratory abnormalities found in humans with HME is the elevation in levels of serum hepatic enzymes. Mildly or moderately elevated hepatic transaminase levels are noted in approximately 80 to 90% of HME patients at some point during their illness, suggesting that there is hepatic injury (32). However, there are relatively few histopathologic data describing lesions in tissues and organs of persons with HME. The previous effort to investigate the pathology of injury in liver tissues from seven patients with laboratory-confirmed HME revealed scattered lobular lymphohistiocytic foci and diffuse lymphohistiocytic infiltration and

Kupffer cell hyperplasia with increased phagocytosis (41). In contrast, the injury in the livers of immunocompetent mice infected with the HF strain progressed rapidly from day 7 to day 9 PI and included Kupffer cell hyperplasia, ballooning cell injury, apoptosis, poorly formed granulomas, erythrophagocytosis, microvesicular fatty metamorphosis, and confluent severe necrosis (29, 43). Cholestasis was observed in 6/7 HME cases, sometimes along with bile duct epithelial injury (41), which was not evident in the previous SCID or SCID/bg models (47), in the present study, or in the immunocompetent mice infected with the HF strain. The causes of the cholestasis associated with HME (26, 41) are unknown.

The pathogenetic mechanisms of hepatic inflammation in HME are unknown. The livers of immunocompetent mice infected with the HF strain are heavily loaded with ehrlichiae (29, 42). However, 4/6 liver tissue samples from humans with HME were not heavily infected with *E. chaffeensis* (41), suggesting that host inflammatory or immune responses contribute to the liver injury seen in HME. The present study extended this notion by showing diverse responses of the liver tissue to *E. chaffeensis* strains with distinct genomic sequences. Leukocyte infiltration in the SCID mice did not correlate directly with bacterial load. For example, the Liberty strain did not induce severe hepatitis despite a higher bacterial load than the Arkansas strain. However, since the Liberty strain retained activity to deplete liver glycogen, it may not be completely inert in the liver. It is tempting to speculate that the hepatitis seen with Wakulla and Arkansas infections may involve a specific factor encoded by an ORF that is absent or mutated in strain Liberty. In this regard, a total of 143 ORFs, including 43 ORFs encoding hypothetical but unknown proteins, were conserved in Wakulla and Arkansas but were different in Liberty. In addition, although both the Arkansas and Wakulla strains induced severe infiltration of inflammatory cells into liver sinusoids, there was a distinct difference in the patterns: Arkansas induced severe granulomatous infiltration, as previously reported (47), whereas Wakulla induced homogeneous and diffuse infiltration disseminating throughout the liver tissue. Focal atrophy and necrosis of liver cell cords were much more pronounced in Wakulla-infected mice than in Arkansas-infected mice. SCID mice retain innate immunity (44). As granuloma formation generally is considered a part of the host innate defense system that can be modified by acquired immunity (46), the Wakulla strain may be able to overcome or suppress this host innate defense.

Glycogen depletion has not been reported in previous studies in patients with HME or in animal models. Glycogen loss in Wakulla- and Liberty-inoculated mice may be due in part to anorexia. However, mice inoculated with Arkansas did not appear to be anorexic at day 15 PI. Glycogen loss in the liver is a typical response to endotoxic shock (24). Since *E. chaffeensis* lacks lipopolysaccharide (20), other ehrlichial components may have contributed to this metabolic disorder in the mice.

As it is impossible for clinical isolates to have the same passage history, one concern is the possibility of mutation and genetic drift during passage of isolates in cell culture or experimental animals. It was reported previously that cell culture passage numbers of *Anaplasma phagocytophilum* influence disease severity in outbred horses but that incubation time, hematological changes, PCR detection, ehrlichial load, serocon-



version time, and titer range do not (36). While this study did not analyze genomic differences between high- and low-passage cultures (36), the present study clearly demonstrated global genomic differences between *E. chaffeensis* strains. Unlike with *A. phagocytophilum* strains with different culture passage numbers (36), the present study revealed striking differences (more than 100-fold) in the bacterial burdens and growth rates among the three *E. chaffeensis* strains. The modest growth of bacteria in mice inoculated with the Arkansas strain was also consistent with the previous data (47). In addition, despite similar low-passage levels in cell culture, the Wakulla and Liberty strains had distinct genomic sequences and induced distinct liver pathologies. Our attempt to revive the Arkansas strain from an early passage received from the CDC was not successful. Nonetheless, the clinical signs and the histopathology of SCID mice in our study were remarkably similar to the previous data for the Arkansas strain reported 9 years ago by others (47). Thus, it is unlikely that the passage history difference is the primary cause of the genomic and phenotypic differences between the three *E. chaffeensis* strains. Regardless of the presence of mutations or drift, in the present study the phenotype and genotype of a given strain are the phenotype and genotype of bacteria with the same passage history.

While the cell-cultured bacteria used in the present study provide a more consistent inoculum than infected tissue (used for uncultivable *Ehrlichia*, such as the HF strain), blood (used for *Anaplasma marginale*, *Ehrlichia ewingii*, and *Anaplasma platys* and for some *A. phagocytophilum* studies), or ticks (used for *A. marginale*, *A. phagocytophilum*, *E. canis*, and *E. ruminantium*), another concern is variations in inocula. In this regard, a previous study showed that the inoculum dose of the Arkansas strain cultured in DH82 cells had a minimal effect on the bacterial load in SCID mice (47). Identification of the molecular basis of these phenotypic differences is perhaps the only way to unequivocally define the nature of virulence of *E. chaffeensis* strains. The present study is the first effort toward this goal.

Use of SCID mice imposes a limitation on the study of acquired immune responses. Recently, *E. chaffeensis* Arkansas cultured in ISE6 tick cells was reported to persist 9 days longer in immunocompetent mice than the same bacteria cultured in DH82 cells persisted (15). Therefore, by finding more suitable culture conditions and *E. chaffeensis* strains more virulent than the Arkansas strain, we may be able to develop an immunocompetent mouse model of *E. chaffeensis* infection that allows investigation of the acquired immune response in the future.

In summary, we demonstrated differences in virulence potential between three *E. chaffeensis* clinical isolates with genomic sequence divergence. Information gained from this study should facilitate many hypothesis-driven studies and characterization of additional strains to help elucidate HME pathogenesis, if all of the tools and advantages of the *E. chaffeensis* genome sequence data and mouse models are used.

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