Erp and Rev adhesins of the Lyme disease spirochete's ubiquitous cp32 prophages assist the bacterium during vertebrate infection

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20 **Abstract**

21 Almost all spirochetes in the genus *Borrelia* (sensu lato) naturally contain multiple
22 variants of closely related prophages. In the Lyme disease borreliae, these prophages are
23 maintained as circular episomes that are called cp32s (circular plasmid 32kb). The cp32s
24 of Lyme agents are particularly unique in that they encode two distinct families of
25 lipoproteins, Erp and Rev, that are expressed on the bacteria's outer surface during
26 infection of vertebrate hosts. All identified functions of those outer surface proteins
27 involve interactions between the spirochetes and host molecules: Erp proteins bind
28 plasmin(ogen), laminin, glycosaminoglycans, and/or components of complement, and
29 Rev proteins bind fibronectin. Thus, cp32 prophages provide their bacterial hosts with
30 surface proteins that can enhance infection processes, thereby facilitating their own
31 survival. Horizontal transfer via bacteriophage particles increases spread of beneficial
32 alleles and creates diversity among Erp and Rev proteins.

33

34 **Introduction**

35 Members of the spirochete genus *Borrelia* (sensu lato) persist through infectious
36 cycles between blood-feeding arthropods and vertebrates, and include agents of
37 infections that plague humans, domestic animals, and wildlife (1, 2). These include Lyme
38 disease, caused by *Borrelia burgdorferi* sensu lato, and tick-borne relapsing fever, caused
39 by *B. hermsii, B. turicatae,* and others. Although a proposal has been raised to divide the
40 genus into *Borrelia* for relapsing fever agents and *Borreliella* for Lyme disease agents, that
41 idea is controversial (3-8). For the purpose of this review, we use the unified genus name
42 *Borrelia*, in part because of the shared property among Lyme disease and relapsing fever
43 spirochetes of near-universal natural colonization with multiple, closely-related cp32
44 prophages (9-13) (Figs. 1 and 2). Of particular significance to this review, the cp32s of
45 both Lyme disease and relapsing fever borreliae carry *mlp* genes, while *erp* and *rev* genes
46 appear to be restricted to the Lyme disease borreliae (11). The significance of those genes
47 will be discussed further below. For simplicity, we also use the name *Borrelia burgdorferi*
48 to encompass all genospecies of Lyme disease spirochetes, *B. burgdorferi* sensu lato.

49 *Borrelia* species all have multipartite genomes, consisting of an approximately one
50 megabase main chromosome and numerous smaller replicons that are generally referred
51 to as "plasmids" (14-16). Some borrelial "plasmids" carry essential genes, and might be
52 better considered to be additional chromosomes (14, 16-20). Another uncommon feature
53 of borrelial genomes is a preponderance of linear DNAs, with the main chromosome and
54 many of the smaller replicons being linear elements with closed hairpin telomeres (14, 16,
55 18, 21-23). Lyme disease spirochetes and many other borreliae also maintain circular
56 DNA replicons (24-26).

57 During the mid-1990s, evidence appeared that *B. burgdorferi* possesses multiple,
58 homologous loci in (26-31). Further studies determined that each related locus is carried

59 on a different circular replicon of approximately 32 kb in size, and that a single bacterial
60 cell can contain numerous different variants of those replicons (9, 26). Based on the sizes
61 and circular nature of the replicons, they were designated "cp32"s (circular plasmid
62 32kb). The majority of nucleotides that comprise different *B. burgdorferi* cp32s are highly
63 conserved, differing significantly at only three loci: one that is responsible for plasmid
64 maintenance and two that encode outer-surface lipoproteins (Fig. 1) (12). The highly
65 repetitive nature of cp32s initially prevented assembly of their sequences, such that the
66 initial, 1997 report of the genome of *B. burgdorferi* type strain B31 did not include the
67 native cp32 plasmids (14). Genomics analysts subsequently used the cp32s as learning
68 tools to refine sequence assembly algorithms, and the entire genome sequence of strain
69 B31, including all cp32s and other repetitive sequences, was finally published in 2000 (16).
70 The different cp32s within a single bacterium appear to be compatible with each
71 other due to their encoding two maintenance proteins, ParA and BpaB, that are distinct
72 for each cp32 within a cell (31-36). Yet, *parA*-*bpaB* locus sequences are conserved between
73 strains (13, 32, 36). Extensive characterization of cp32 sequences from Lyme disease
74 spirochetes that were collected around the world revealed twelve apparent clades of cp32
75 *parA*-*bpaB* pairings (13, 36, 37). Additionally, some strains contain small plasmids of
76 approximately 9 kb that were derived from cp32s and which carry only a *bpaB* allele (14,
77 16, 38). The absence of a *parA* gene from those cp9 plasmids raises intriguing questions
78 about plasmid maintenance in *Borrelia* species.
79 The conserved size and sequence of *B. burgdorferi* cp32s, along with genes for
80 proteins that resemble bacteriophage portals and holins, led to an early hypothesis that
81 they might be prophage genomes (9, 39). Cultures of *B. burgdorferi* have been observed to
82 clear spontaneously, or when exposed to 1-methyl-3-nitroso-nitroguanadine (MNNG),

83 with production of bacteriophage particles that were named ϕBB1 (10, 40). Molecular
84 analyses of those particles revealed that they contained cp32 DNA, and that they were
85 capable of transducing nucleic acids between *B. burgdorferi* cells (41, 42). A subsequent
86 study revealed that MNNG can induce transcription of a long, multigene cp32 operon
87 that presumably encodes bacteriophage structural proteins (43). Intriguingly, even
88 though MNNG is a mutagen, *B. burgdorferi* lacks an SOS system (14), so the means
89 through which this chemical induces bacteriophage production is not yet known. It was
90 recently reported that dysregulation of the RpoS alternative sigma factor led to
91 production of ϕBB1 bacteriophage particles (44). Characterizing the mechanism that
92 controls the lysogenic vs. lytic phases of ϕBB1 will undoubtedly yield important insights
93 on the breadth of bacteriophage biology.
94
95 ***B. burgdorferi* cp32-encoded outer-surface Erp lipoproteins.**
96 Two antigenic outer-surface proteins of *B. burgdorferi* strain N40 were reported in
97 1994 and named OspE and OspF (outer surface proteins E and F) (45). Shortly afterward,
98 we found that *B. burgdorferi* N40 expresses different levels of those two proteins under
99 different culture conditions (46). Screening of a lambda library of *B. burgdorferi* type strain
100 B31 DNA revealed several distinct loci in that strain that were closely related to, but
101 distinct from, the strain N40 *ospE-ospF* locus (26). As none of the strain B31 loci could be
102 definitively called "*ospE-ospF*", they were designated "*erp*" (OspEF-related protein).
103 Further analyses revealed that various clonal cultures of *B. burgdorferi* B31 contain up to
104 9 different *erp* loci (9, 16) (Table 1). Strain N40 contains 6 *erp* loci, including the initially-
105 described *ospE-ospF* (13, 36, 37, 45, 47, 48) (Table 1). Researchers who investigated other
106 strains discovered additional *erp* genes, and gave them a variety of names such as *p21,*

107 *bbk2.10*, *bbk2.11*, *pG* and *elp* (28, 29, 47, 49-53) (Table 1). Naming of these genes and
108 proteins by multiple groups has, unfortunately, led to a rather chaotic nomenclature.
109 Nonetheless, to avoid further confusion, we use the names originally applied to these
110 genes and proteins, and urge other authors to also retain names that have been in
111 common use for the past several decades. Structures have been determined for some Erp
112 proteins, primarily alleles that occur in the B31 type strain (54-57).

113 Considering *erp* genes and their proteins as a unified group is supported by their
114 all occupying an allelic location on cp32s, their conserved promoter and operator DNA
115 sequences, simultaneous co-expression in culture and during infection processes,
116 possession of conserved leader polypeptides and initial residues of their mature
117 lipoproteins, and localization of the mature lipoproteins on the outside of the borrelial
118 outer membrane (12, 58). Evolutionary analyses indicated that *erp* genes arose from a
119 single sequence that encodes the leader polypeptide and the 5 amino acid sorting
120 sequence, while the remainder of the open reading frames descended from a large
121 number of independent origins that frequently undergo novel recombination events (36).

122 Dendrograms of known *erp* sequences show at least three branches (32, 36, 59),
123 which led to a suggestion that *erp* genes could be divided and given three different group
124 names: *ospE*, *ospF*, and *elp* (59). In addition to variations in nucleotide sequences, the
125 encoded proteins generally differ considerably in size, with OspE-types being small
126 (approximately 20 kDa), OspF-types being medium sized (approximately 25-30 kDa), and
127 Elp-types being large (approximately 40 kDa) (36). We do not find the OspE/OspF/Elp
128 nomenclature to be particularly helpful, since functions are not always conserved within
129 each group, and there are no consistent differences in gene expression or operon
130 structures. In addition, due to frequent natural recombination events having occurred
131 between *erp* genes, some extended nucleotide sequences are found in members of all three

132 groups (36). For example, some "*elp*" alleles share greater similarities with "*ospF*" genes
133 (as high as 34%) than they do with other "*elp*" genes (as low as 17%) (36). Recombination
134 events have also resulted in alleles that do not fit in the tripartite scheme (36). Extensive
135 analyses of *erp* open reading frames, operon structures, promoter sequences, and both
136 intragenic and intergenic recombination, indicated that *erp* genes evolved from a single
137 promoter/leader/localization sequence and a large number of diverse sequences that
138 encode the external parts of the proteins (36).

139 Erp lipoproteins are localized to the borrelial outer surface (45, 60, 61). The strain
140 B31 Erp proteins are all simultaneously co-expressed, consistent with their genes
141 possessing conserved promoter and operator sequences (62-64). Differences in gene or
142 protein expression have been reported for two other strains, but the reasons for those
143 apparent variations were not investigated (47, 61, 65). The mechanism by which *B.*
144 *burgdorferi* controls transcription of *erp* operons has been well studied, and shown to
145 involve interactions between the BpaB repressor, BpuR co-repressor, and EbfC
146 antirepressor proteins that bind to specific sites adjacent to *erp* promoters. There is
147 evidence that some *erp* operons may be affected by an alternative sigma factor, RpoS (66,
148 67). For an extensive review of the mechanisms by which *erp* transcription is regulated,
149 please see our recent review on that topic (68).

150 *B. burgdorferi* expresses little to none of any Erp protein during colonization of
151 unfed ticks (47, 63, 69). As the tick begins to feed on the blood of a vertebrate host, the
152 bacteria induce production of their entire repertoire of Erp proteins (63). Indirect
153 fluorescence analyses (IFAs) of skin samples at the sites of tick attachment revealed that
154 almost all transmitted *B. burgdorferi* produced detectable levels of every examined Erp
155 protein (63). Quantitative reverse transcription PCR (qRT-PCR) and promoter fusions
156 with *gfp* revealed that *erp* genes remain induced throughout persistent mammalian

157 infection (53, 63, 70). Levels of antibodies against Erp proteins, including IgM, remain
158 elevated during prolonged infection of mice (53, 63, 70-72). The persistence of IgM
159 antibodies against Erp proteins may reflect *B. burgdorferi*'s ability to manipulate host
160 immune responses (73-76). That phenomenon may also explain how *B. burgdorferi* is able
161 to persist extracellularly despite having its outer surface covered with Erp, Rev, and other
162 antigenic proteins. IFAs of naive ticks feeding on infected mice showed that *B. burgdorferi*
163 continue to produce Erp proteins during acquisition by feeding ticks, which ceases soon
164 after detachment (63).

165 Numerous functions have been identified for Erp proteins, all of which involve
166 interactions with vertebrate host tissues:

167 Several Erp proteins, including ErpA, ErpC, and ErpP of strain B31 and OspE of
168 strain N40, exhibit high affinities for complement factor H and the related proteins FHL-
169 1 and the FHR group (71, 77-91). Binding factor H and FHL-1 to *B. burgdorferi* via Erp
170 proteins confers resistance to the alternative pathway of complement activation. The
171 exact functions of FHR proteins are unclear, but they also appear to be involved with
172 complement control (92). Erp proteins with different sequences show varying affinities
173 to factor H molecules of different potential vertebrate host species, and may thus play
174 roles in expanding the host range of Lyme disease spirochetes (85, 93).

175 The ErpB, ErpM and ErpQ proteins of strain B31 (confusingly called ElpB, ElpM
176 and ElpQ in the publications) were recently demonstrated to bind complement factors
177 C1s and C1r (94, 95). Adhesion of those host proteins onto the borrelial surface appears
178 to afford protection from the classical arm of complement.

179 The ErpX protein of *B. burgdorferi* B31 binds human laminin (96). This may help
180 the spirochete target and/or adhere to laminin-rich tissues. The laminin-binding domain
181 of ErpX was mapped to a central, disordered domain that is almost entirely comprised of

182 charged amino acids (96). Of note, other strain B31 Erp proteins with amino acid
183 sequences that are similar to ErpX (others in the "Elp" group: ErpB, ErpM and ErpQ,
184 Table 2) do not detectably bind human laminin (96).

185 The *B. burgdorferi* B31 ErpG and six similar proteins from other strains bind
186 heparan sulfate, and promote attachment to glial cells (97). A phage display library,
187 injected into mice and screened for potential *B. burgdorferi* adhesins, found clones of B31
188 ErpG, ErpK, and ErpL, and strain 297 Bbk2.10 in mouse heart, bladder, and tibiotarsal
189 joint tissues (98). Those four proteins share some sequence similarities (Table 2) (36).

190 The *B. burgdorferi* B31 ErpA, ErpC, and ErpP proteins bind host plasminogen and
191 its proteolytically-active form, plasmin (99). Plasmin bound to Erps and other surface
192 proteins facilitates *B. burgdorferi* migration through solid tissues in vitro, a characteristic
193 that is hypothesized to also enable dissemination during vertebrate infection (100-109). It
194 is notable that the Erp proteins that bind plasmin(ogen) also bind complement factor H
195 (see above).

196 Due to the surface localization and antigenicity of Erp proteins, there have been
197 several investigations into their use as protective vaccines or for serological diagnosis of
198 *B. burgdorferi* infection. However, vaccination of mice with the OspE, OspF, or P21
199 proteins of strain N40 did not provide substantial protection against infection by the same
200 strain (47, 69). Several recombinant Erp proteins have been examined for serological
201 diagnosis of human infection, with mixed results (110-113).

202

203 ***B. burgdorferi* cp32-encoded outer-surface Rev and Mlp lipoproteins.**

204 The cp32 replicons of Lyme disease and relapsing fever borreliae carry a distinct
205 mono- or bicistronic *mlp* (multicopy lipoprotein) operon (Figs. 1 and 2) (11, 16, 30). In the
206 majority of Lyme disease spirochete cp32s, the *mlp* locus is preceded by a distinct gene,

207 *bdr*, which has its own promoter (16, 30) (Fig. 1). The characterized cp32 of the relapsing
208 fever spirochete *B. hermsii* contains a single *mlp* gene, without an adjacent *bdr* (11).
209 Curiously, all cp32s of Lyme disease and relapsing fever borrelia carry a second *bdr* allele
210 adjacent to the episomal replication locus (9, 16, 31) (Fig. 1). Bdr proteins are associated
211 with the bacterial inner membrane, are produced during vertebrate infection, but their
212 function(s) have yet to be discovered (72, 114-122).

213 For unknown reasons, some *B. burgdorferi* cp32s do not contain a *bdr* adjacent to
214 the *mlp* locus, but instead carry a distinct gene named *revA* (so named because such genes
215 are arranged <u>rev</u>erse to *mlp*) (16, 30). The *revA* ORF and promoter sequences are very
216 different from those of *mlp* genes, implying that *mlp* and *revA* loci are genetically
217 unrelated (9, 16, 30). In the *B. burgdorferi* type strain, B31, cp32-1 and cp32-6 each contain
218 a *revA* gene adjacent to their *mlp*, while all other B31 cp32s contain only a *mlp* gene (16).

219 Mature RevA is a lipoprotein and localizes to the *B. burgdorferi* outer surface (123).
220 Additionally, some truncated cp32 derivatives, such as B31 cp9, carry a similar gene
221 named *revB* (14, 16, 123). The two *revA* orthologs of strain B31 encode an identical RevA
222 protein, while that strain's RevB protein shares only 28% amino acid identity with RevA.
223 Other strains encode RevA and RevB proteins with varying degrees of differences (30).

224 *B. burgdorferi* in unfed ticks do not produce detectable levels of *revA* mRNA, but
225 do produce elevated transcripts during mouse infection (124). Human patients and
226 experimentally infected mice produce antibodies against RevA (125-127). In culture, *revA*
227 transcription is induced by changes in temperature or pH that correspond to changing
228 conditions encountered by the bacteria during tick feeding and mammalian infection
229 (123). Despite its antigenicity and surface localization, vaccination with RevA did not
230 protect mice from *B. burgdorferi* infection (128).

231 *B. burgdorferi* B31 RevA binds the 70kDa N-terminal region of fibronectin via a
232 domain in the RevA N-terminus (124). This binding was not affected by salt or heparin,
233 suggesting the interaction of these two proteins involves more than ionic interactions.
234 The *B. burgdorferi* RevB protein also binds to fibronectin, despite having limited sequence
235 identity to RevA (124). Polyclonal antibodies against RevA did not block adherence of
236 fibronectin to wild type *B. burgdorferi*, although those antibodies did prevent fibronectin
237 adherence to *B. burgdorferi* that were deleted of another fibronectin-binding protein,
238 BBK32 (129, 130). Similarly, binding of wild-type *B. burgdorferi* to HUVECs or human
239 neuroglial cells was not blocked by anti-RevA monoclonal antibodies (131). Those results
240 underscore the functional redundancy of *B. burgdorferi* fibronectin-binding proteins (132).
241 RevA also binds mammalian laminin, although to a significantly lesser extent than to
242 fibronectin (124).

243 A *B. burgdorferi* mutant unable to produce RevA was found to be defective in
244 colonization of cardiac tissue (130). That mutant also caused increased pathology of
245 tibiotarsal joints and enhanced CCL2 production. Increased inflammation in the absence
246 of RevA protein suggests a role for that surface protein in immune evasion, perhaps by
247 masking a more immunogenic antigen.

248 Precise functions have not yet been ascribed to Mlp proteins. The cp32 prophages
249 of both Lyme disease and relapsing fever *Borrelia* spp. carry *mlp* genes, which suggests
250 that the proteins serve functions that are conserved among borreliae (9, 11, 16, 30, 133)
251 (Fig. 2). As with *erp* and *rev* genes, there is often considerable sequence diversity among
252 *mlp* genes within and between *B. burgdorferi* isolates (16, 133, 134). The patterns of
253 regulated expression and antigenicity of *B. burgdorferi* Mlp proteins imply that they serve
254 functions within vertebrate hosts (134-139). A recombinant Mlp protein was found to
255 adhere to human brain microvascular endothelial cells (140). The structure of the

256 MlpA/P28 protein that is encoded by B31 cp32-1 has been solved, and was noted to
257 contain a structural motif that is similar to one seen in a lipid-binding protein (16, 134,
258 141).
259
260 **Horizontal transfer and recombination**
261 Almost all examined Lyme disease spirochete isolates naturally contain 6-12
262 distinct cp32s, each with an *erp* and a *mlp* / *revA* locus. As noted above, relationships
263 between cp32s can be discriminated by their *parA-bpaB* maintenance loci. Using that as
264 reference, there is clear evidence of recombination having occurred between cp32s, and
265 exchange of cp32 DNA between bacteria (13, 32, 36, 37).
266 The original isolate of *B. burgdorferi* type strain B31 carried three identical *erpAB*
267 operons on cp32-1, cp32-5, and cp32-8 (9, 26). In contrast, those cp32s of other strains
268 possess distinct *erp* operons (32, 33, 36). The cp32-1 and cp32-6 of strain B31 carry *revA*
269 genes that are nearly identical in sequence, and which encode identical RevA proteins,
270 while related plasmids of other strains have different genes at that locus (16, 124, 127).
271 Two other recombination events are also evident in strain B31: cp32-4 has an fragment of
272 a cp32 *sbbP* gene inserted into *erpH*, such that a complete lipoprotein cannot be produced,
273 and cp32-3 has an unrelated gene, *bapA*, inserted 3' of *erpG* (9, 26). No other B31 cp32
274 carries a *bapA* gene, although paralogous genes, named *eppA*, are found on some cp9
275 replicons (142-144).
276 Despite cp32s having nearly identical sequences, and multiple cp32s residing
277 within individual bacteria, there are no known examples of recombination occurring
278 during cultivation. Recombination does not take place during vertebrate infection, or, at
279 least, not among bacteria of the same strain (145, 146). A report suggesting that variation
280 arose during mammalian infection was subsequently seen to be based on PCR artifacts

281 (146, 147). We hypothesize that genetic transfer and recombination occur when *Borrelia*
282 spirochetes are within ticks. A recent study of borrelia bacteriophage induction supports
283 that hypothesis (44).

284 Additional evidence of horizontal transfer of cp32 DNAs in nature was obtained
285 from sequencing genomes of multiple isolates (36). *B. burgdorferi* strains B31 and BL206
286 contain almost identical repertoires of cp32s, the only differences being presence of a
287 cp32-1 in only B31 and a cp32-11 in only BL206. Intriguingly, B31 cp32-1 and BL206 cp32-
288 11 carry an identical *erpAB* locus. Two other strains, 297 and Sh-2-82, contain cp32
289 repertoires that are identical to each other, with the addition of a cp32-8 in only Sh-2-82.
290 The cp32-8 *erp* locus of Sh-2-82 is identical to the cp32-8 *erpAB* locus of strain B31,
291 implying that a cp32-8/ϕBB1 was exchanged between ancestors of those two strains.
292 Strain B31 was isolated from a tick on Shelter Island, New York, in 1981, and Sh-2-82 was
293 isolated from another tick on the same island in 1982 (148, 149). It is also notable that
294 BL206, which is a near clone of B31, was isolated several years later from the blood of a
295 Lyme disease patient in Westchester County, New York (150), and that 297, which is a
296 near clone of Sh-2-82, was isolated from the cerebrospinal fluid of another patient (151).

297 Transduction of cp32 DNA between different strains of *B. burgdorferi* has been
298 observed in culture (41). The mechanisms that induce ϕBB1 particles to form are not yet
299 known. As noted above, *B. burgdorferi* does not possess an SOS system (14). The timing
300 of bacteriophage production is also unknown. All *Borrelia* species are obligate parasites
301 of arthropods and vertebrates, so triggers for ϕBB1 would be limited to conditions
302 experienced within those hosts. Ticks can acquire multiple strains of *B. burgdorferi*, which
303 would reside together in the tick's midgut, leading us to propose that the midguts of
304 feeding ticks are likely locations for DNA exchange. Tick feeding is a time of rapid

305 replication for *B. burgdorferi*, which appears to be used by the spirochetes as an important
306 cue that transmission is imminent (68, 152). That would also be an appropriate time for
307 bacteria to exchange DNA and diversity their repertoires of Erp, Rev, and other surface
308 proteins that interact with vertebrate hosts.

309 At least two advantages appear to be conferred upon *B. burgdorferi* by exchanging
310 and recombining *erp* and *rev* genes. First, both groups of proteins are expressed early
311 during vertebrate infection, are located on the bacterial outer surface, and are antigenic.
312 Reservoir vertebrates are generally fed upon by numerous ticks, all of which might be
313 infected. Thus, Lyme disease spirochetes are under selective pressure to diversify
314 sequences of early, surface-borne antigens, to facilitate infection of animals that have
315 previously been exposed. A second advantage to Erp and Rev variability among
316 individual bacteria stems from their functions as adhesins, and from the fact that the ticks
317 that transmit Lyme borreliae may not be specific in their choices of hosts. For example, in
318 the northeastern USA, *Ixodes scapularis* ticks feed on birds, mice, and other mammals
319 (153). Since a *B. burgdorferi* cannot "know" in advance what species of vertebrate its tick
320 vector will feed upon, it would be advantageous for a bacterium to enter its new host
321 while bristling with a wide variety of adhesins that differ in relative affinities for ligands
322 of different hosts, with the likelihood that some Erp, Rev, and other adhesins will be
323 adequate for the bacteria's survival.

324

325 **Exceptional *Borrelia***

326 To date, only two exceptions are known to the generalization that *Borrelia* species
327 naturally carry cp32s. The agent of avian spirochetosis, *Borrelia anserina*, is the type
328 species of the genus, and lacks cp32 DNA (11). Since Lyme disease and relapsing fever

329 *Borrelia* species carry cp32s, their absence from *B. anserina* suggests that either ϕBB1
330 entered in the genus after the split of *B. anserina* from the other species, *B. anserina* has a
331 means to exclude cp32s/ϕBB1, or cp32s encode properties that are beneficial to Lyme
332 disease and relapsing fever borreliae, but not to avian spirochetosis borreliae. The second
333 known example of a cp32-deficient *Borrelia* is strain Far04, a Lyme disease spirochete that
334 was isolated from a puffin that was collected on the Faroe Islands, Denmark (13, 154, 155).
335 The densely packed puffin nests on those islands are homes to *Ixodes uriae* ticks, which
336 preferentially feed on seabirds. Although Far04 is a single example, it is possible that *I.*
337 *uriae* ticks on the islands primarily feed on puffins, to the extent that some circulating
338 borreliae have lost the need for the variability of adhesin compositions afforded by cp32
339 prophages. Clearly, additional sampling and analyses are needed to test that hypothesis.
340

341 **Future research directions.**

342 Erp and Rev lipoproteins localize to the borrelial outer surface, and are known to
343 interact with a variety of vertebrate host proteins that protect against immune responses
344 and facilitate tissue invasion and colonization. Noting that Erp and Rev proteins were
345 discovered during the mid-1990s, and that adherence of complement C1r and C1s was
346 first reported in 2022, we consider it likely that other functions remain to be discovered.
347 Additional questions include: Why do the cp32s of Lyme disease spirochetes encode Erp
348 and Rev proteins, while those of relapsing fever spirochetes appear to lack those genes?
349 What are the functions of the Mlp outer surface proteins, which are encoded on cp32s of
350 both Lyme disease and relapsing fever borreliae? Why does *B. anserina*, the agent of avian
351 spirochetosis, lack cp32s? When do borreliae produce ϕBB1 bacteriophages during their
352 natural infectious cycles? There is genetic evidence of DNA transfer of other genes

353 encoding borrelial antigens, such as outer surface protein C (OspC) (156, 157) – might
354 that occur by generalized transduction via ϕBB1?
355
356 **Concluding statements**
357 The cp32 prophages / ϕBB1 bacteriophages have evidently co-evolved with many
358 species of *Borrelia*. They have acquired a number of different genes that confer benefits to
359 their bacterial hosts, which thereby enhance their own survival. These bacteriophages
360 have adopted a primarily lysogenic lifestyle, with a limited degree of bacteriophage
361 particle production and host cell lysis. The *parA* and *bpaB* maintenance genes have
362 diversified extensively, to the extent that large numbers of different cp32s can be
363 maintained in a single bacterial cell. This provides both extensive repertoires of *erp*, *rev*,
364 and other genes for horizontal exchange and enables the host bacteria to express wide
365 varieties of surface adhesins during vertebrate infection.
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368
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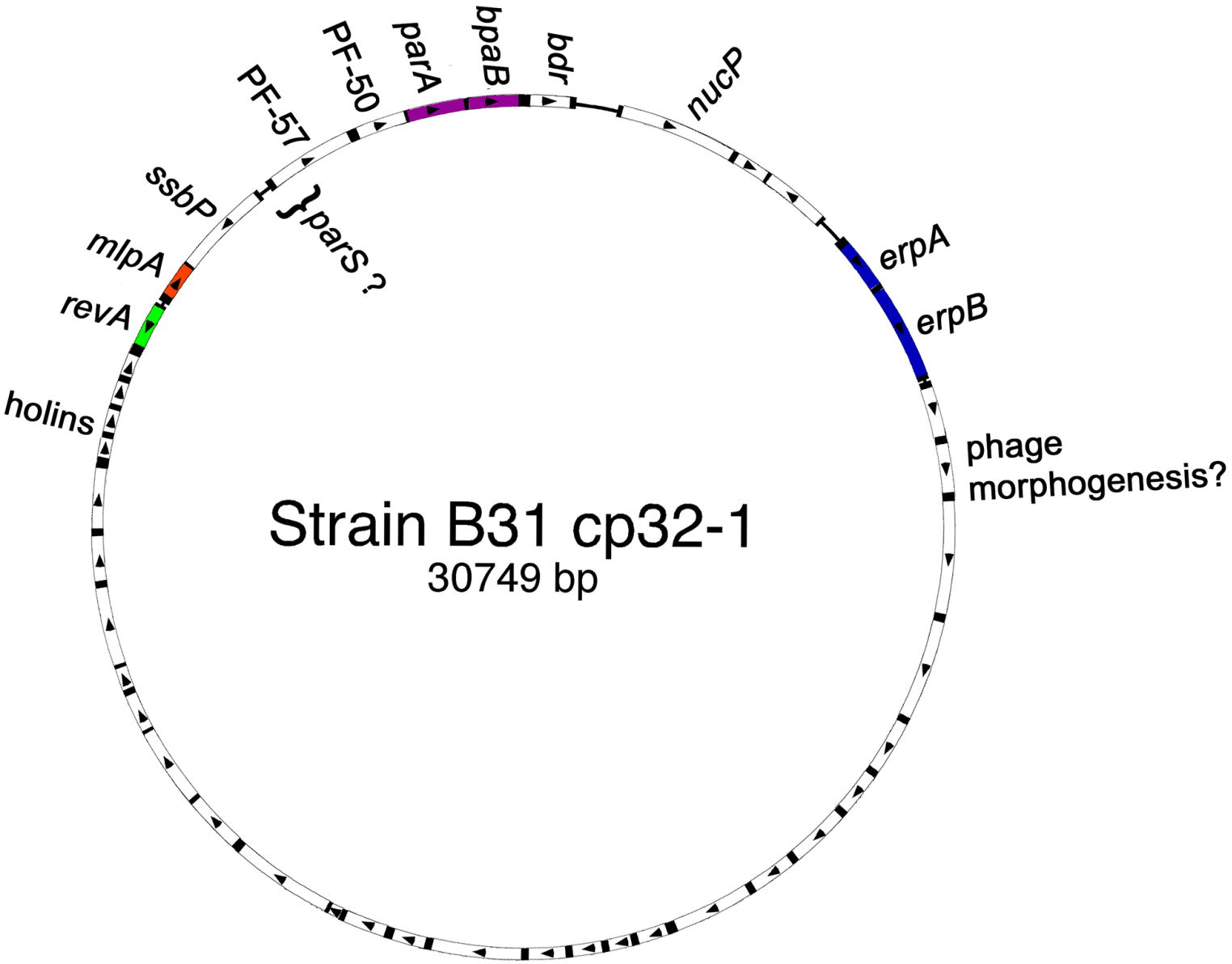
374

375 **Figure 1.** Schematic of the open reading frames of a representative *B. burgdorferi* cp32, the
376 cp32-1 of type strain B31. Arrowheads indicate direction of open reading frame
377 transcription. *B. burgdorferi* cp32s contain two loci that encode lipoproteins that are
378 expressed on the bacterial outer surface: a mono- or bicistronic locus that encodes Erp
379 proteins, and a separate locus that consists of either divergently-transcribed *revA* and *mlp*
380 genes, or a *mlp* operon adjacent to a *bdr* gene. B31 cp32-1 and cp32-6 each carry a *revA*
381 gene, while the other cp32s of that strain instead carry a *bdr* gene adjacent to their *mlp*
382 locus. Borreliae are apparently able to maintain numerous, different cp32s in a single cell
383 due to diversity among the *parA-bpaB* (borrelial *parB*-analog) locus. Other open reading
384 frames show up to 100% conservation between different cp32s and *B. burgdorferi* strains,
385 shown as white. Two conserved genes, PF-50 and PF-57, are located adjacent to the *parA-*
386 *bpaB* loci of all cp32s and are necessary for replicon maintenance (158-160). The *parS*
387 segregation site appears to be associated with the 5′ end of *PF-57* (our unpublished results
388 and (158-160). Conserved genes *ssbP* and *nucP* encode a single-stranded DNA binding
389 protein and nuclease, respectively (35). Bacteriophage structural and assembly proteins
390 appear to be encoded from a single operon that extends from 3′ of the *erp* locus to the
391 holin-encoding genes (16, 39, 43, 161).
392
393 **Figure 2.** Alignment of the open reading frames of *B. burgdorferi* B31 cp32-1 and a
394 composite cp32 of *B. hermsii* HS-1 that was assembled from sequenced contigs. Directions
395 of transcription are indicated by arrows. The *erp*, *revA* and *mlp* genes are indicated by
396 hatching. ORFs that are nearly identical between cp32s of *B. burgdorferi* and *B. hermsii* are
397 indicated by white boxes. Note that the plasmid replication genes, including *parA* and
398 *bpaB*, are located in reversed directions on the two different cp32 types. Adapted from
399 (11).


bpaB
bdr
nucP
parA
PF-50
PF-57
ssbP
parS ?
mlpA
revA
holins
erpA
erpB
phage
morphogenesis?
Strain B31 cp32-1
30749 bp

B. burgdorferi B31
cp32-1
B. hermsii HS1
cp32 composite
holins
revA
mlp
ssbP
PF-57
PF-50
parA
bpaB
bdr
nucP
erpA
erpB
holins
ssbP
bdr
bpaB
parA
PF-50
PF-57
nucP
mlp

400 **Table 1:** The cp32 DNA elements and associated *erp* loci that occur in the three best-
401 characterized Lyme disease spirochete isolates: *B. burgdorferi* strains B31, N40, and 297.
402 The *erp* nomenclature has been applied to the majority of other characterized strains of
403 Lyme disease spirochetes (36, 88). Plasmid nomenclature is standardized according to
404 segregation locus type, such that all plasmids designated "cp32-1" contain a similar
405 segregation locus, etc. All known cp32 segregation loci fall into 12 clades. The first *B.*
406 *burgdorferi* strain to be completely characterized, B31, contains 2 distinct cp32 prophages
407 that possess identical maintenance loci, cp32-2 and cp32-7, and define segregation locus
408 type 32-2/7. As a result, the numbering scheme for the 12 groups goes up to cp32-13. The
409 published names of *erp* genes are listed. A variety of naming schemes were applied to
410 these genes by their discoverers, particularly for strain 297, which has led to a sometimes
411 confusing nomenclature. Additional *erp* genes of strain N40 that were discovered by our
412 group were given allele numbers only (e.g. *erp22, erp23*, etc.). Other strain's *erp* genes have
413 been designated either by an allele number (with a different number for each gene) or by
414 genome sequencing ORF numbers, which are too numerous to list here but examples are
415 described in (36).
416

	B. burgdorferi strain		
cp32 segregation locus type	B31 [a]	N40 [d]	297 [e]
cp32-1	*erpA1, erpB1* (or *erpB2*) [b]		*ospE1, elpB1-1*
cp32-2/7	*erpC, erpD*	*ospE, ospF*	*elpA2*

	erpL, *erpM* [c]		
cp32-3	*erpG*		*ospF*
cp32-4	*erpH*, *erpY*	*erp23*, *erp24*	*elpA1*
cp32-5	*erpA5*, *erpB5* (previously *erpI*, *erpJ*)	*erp25*	*ospE5*, *elpB1-5*
cp32-6	*erpK*		*bbk2.10*
cp32-8	*erpA8*, *erpB8* (previously *erpN*, *erpO*)		
cp32-9	*erpP*, *erpQ* [c]	*p21*, *erp22*	*p21*, *elpB2*
cp32-10	*erpX*	*erp26*	
cp32-11			*bbk2.11*
cp32-12		*erp27*	*ospE12*, *elpB1-12*
cp32-13			

417

418 a. The *erp* loci of strain B31 were first identified and named by the authors and their
419 research colleagues (9, 16, 26). Strain B31 was one of the first organisms to have its
420 genome completely sequenced, but the sequenced culture lacks cp32-2 and cp32-5, so the
421 complete sequences of those prophages remains uncertain. Prophages cp32-1, cp32-5, and
422 cp32-8 carry identical *erp* loci, which are distinguished by allele numbers. The *erp* loci of
423 cp32-1, cp32-5, and cp32-8 encode identical proteins, which are collectively called ErpA
424 and ErpB. At some time in the past, the B31 cp32-10 underwent integration into an
425 unrelated linear replicon, which created an approximately 56kb linear plasmid that is
426 named lp56.

427

428 b. Two alleles of *erpB* have been identified in different cultures of strain B31. The *erp* locus
429 that was first identified in a high-passage, non-infectious culture contains a premature
430 stop codon within the *erpB* gene, and thus encodes a truncated protein (26). Subsequent
431 analyses of infectious cultures of B31 revealed a full-length *erpB* open reading frame,
432 which was designated allele *erpB*2 (9, 16).
433
434 c. Two recent publications designated ErpB as "ElpB", ErpM as "ElpM" and ErpQ as
435 "ElpQ" (94, 95).
436
437 d. Two *erp* loci of strain N40, *ospEF* and *p21*, were named by Fikrig and colleagues (45,
438 47). The remainder of the N40 genes were discovered and named by the authors and their
439 coworkers (36). The cp32-2/7 prophage of strain N40 is naturally truncated to an
440 approximately 18kb circular replicon, cp18 (48).
441
442 e. The *erp* loci of strain 297 were discovered and named by Radolf and colleagues (28, 33,
443 59). Our research group later found that the cp32-1, cp32-5, and cp32-12 prophages each
444 contain an identical *erp* locus, *ospE-elpB1*, which we differentiated by additional allele
445 numbers (36). The cp32-2/7 and cp32-9 plasmids of strain 297 are naturally truncated,
446 and have been named cp18-a and cp18-2, respectively (33).
447
448
449

450 **Table 2.** The Erp proteins of the three best-characterized *B. burgdorferi* strains, B31, N40,
451 and 297, sorted according to the tripartite OspE/OspF/Elp scheme. Other characterized
452 strains of Lyme disease spirochetes have generally applied the *erp* nomenclature (36, 88).
453 Please note that even though some proteins of strain 297 proteins were assigned the same
454 names as N40 proteins, those proteins are distinct from each other. That is, N40 OspE is
455 not identical to 297 OspE, N40 P21 is not identical to 297 P21, and N40 OspF is not
456 identical to 297 OspF. The strain 297 nomenclature is further complicated in that the 297
457 OspE protein is more similar to the N40 P21 protein than to the N40 OspE protein, and
458 the 297 P21 protein is more similar to the N40 OspE protein (36).

	"OspE" "small" (69% average pairwise identity) [a]	"OspF" "medium" (44% average pairwise identity) [a]	"Elp" "large" (40% average pairwise identity) [a]
B. burgdorferi B31	ErpA ErpC ErpH ErpP	ErpG ErpL ErpK ErpY	ErpB ErpD ErpM ErpQ ErpX
B. burgdorferi N40	OspE P21	OspF Erp23 Erp25 Erp27	Erp22 Erp24 Erp26
B. burgdorferi 297	OspE P21	OspF Bbk2.10	ElpA1 ElpA2

		Bbk2.11	ElpB1 ElpB2

459

460 a. Average pairwise identities of genes and proteins of 21 analyzed Lyme disease *Borrelia*
461 strains (36). That study found that some alleles that fall into the OspF group due to their
462 sizes share greater sequence identity with members of the Elp group than they do with
463 other members of the OspF group, and vice versa. Those similarities and differences
464 appear to be due to frequent recombination between alleles of different groups.

465

466

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