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1 **ORF4 of the temperate archaeal virus SNJ1 governs the lysis-lysogeny switch**
2 **and superinfection immunity**

3

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15

16 **Abstract**

17 Recent environmental and metagenomic studies have considerably increased the
18 repertoire of archaeal viruses and suggested that they play important roles in nutrient
19 cycling in the biosphere. However, very little is known about how they regulate their
20 life cycles and interact with their hosts. Here, we report that the life cycle of the
21 temperate haloarchaeal virus SNJ1 is controlled by the product ORF4, a small protein
22 belonging to the antitoxin MazE superfamily. We show that ORF4 controls the
23 lysis-lysogeny switch of SNJ1 and mediates superinfection immunity by repression of
24 genomic DNA replication of the superinfecting viruses. Bioinformatic analysis shows
25 that ORF4 is highly conserved in two SNJ1-like proviruses, suggesting that the
26 mechanisms for lysis-lysogeny switch and superinfection immunity are conserved in
27 this group of viruses. As lysis-lysogeny switch and superinfection immunity of
28 archaeal viruses are poorly studied, we suggest that SNJ1 could serve as a model
29 system to study these processes.

30

31 **Importance**

32 Archaeal viruses are important parts of the virosphere. Understanding how they
33 regulate their life cycles and interact with host cells provide crucial insights into their
34 biological functions and the evolutionary histories of viruses. However, mechanistic
35 studies of the life cycle of archaeal viruses are scarce due to a lack of genetic tools
36 and demanding cultivation conditions. Here, we discover that the temperate
37 haloarchaeal virus SNJ1, which infects *Natrinema* sp. J7, employs a lysis-lysogeny

38 switch and establishes superinfection immunity like bacteriophages. We show that its
39 ORF4 is critical for both processes and acts as a repressor of the replication of
40 SNJ1. These results establish ORF4 as a master regulator of SNJ1 life cycle and
41 provides novel insights on the regulation of life cycles by temperate archaeal viruses
42 and on their interactions with host cells.

43

44 **Introduction**

45 The study of archaeal viruses is still in its infancy compared to the widely reported
46 bacteriophages (about 8,500 species) (1). Only about 120 species of archaeal viruses
47 have been sequenced and investigated to date; however, they display remarkable
48 diversity in virion morphologies and gene contents (2-4). Temperate life cycles are
49 common among viruses of bacteria and archaea, whereby viral genomes are stably
50 maintained within the cell either as extrachromosomal episomes or are integrated into
51 the host chromosomes. This mode of propagation provides ample opportunities for the
52 virus and its host to co-evolve (5, 6). Therefore, research on the viral life cycle and the
53 interaction between temperate viruses and their hosts can provide crucial insights into
54 the evolutionary histories of viruses and novel modes of virus-host interactions (7, 8).
55 However, due to the lack of suitable genetic tools and demanding cultivation
56 conditions, only a handful of archaeal temperate viruses have been studied
57 experimentally so far (9-17).

58 Unlike temperate archaeal viruses, temperate bacteriophages are ubiquitous and the
59 regulation of their life cycle has been extensively studied (18-20). A logical parallel
60 may be drawn from these studies: temperate viruses have a dual life cycle, which can
61 terminate either in host cell lysis or lysogeny. If the lytic pathway is chosen, the virus
62 actively replicates its genome, produces progeny virions and lyses the host cell. By
63 contrast, if the lysogenic pathway ensues, the viral genome will be present in a
64 dormant state either as an extrachromosomal element or as a provirus integrated into
65 the host chromosome, and the expression of the genes responsible for the lytic

66 pathway is repressed. Temperate viruses often confer immunity to their host cells
67 against subsequent infection by the same and closely related viruses, a phenomenon
68 known as superinfection immunity (21, 22). When host cell fitness decreases, such as
69 upon exposure to DNA damaging agents, the provirus switches to the lytic pathway
70 (23). The switch between the lytic and lysogenic states of temperate bacteriophages is
71 very sophisticated and is normally controlled by a master regulator which senses the
72 fitness of the host cell and represses the expression of the lytic pathway genes. The *cI*
73 protein of the *Escherichia coli* phage λ is a prototypical master regulator which
74 represses the expression of the lytic pathway genes and is cleaved under conditions of
75 DNA damage (23-26). Among archaeal viruses, the switch between lytic and
76 temperate life cycles has been explored in the case of the spindle-shaped virus SSV1
77 infecting hyperthermophilic archaea of the genus *Sulfolobus* (15-17, 27). It has been
78 suggested that SSV1 protein F55 binds to host protein RadA and this complex
79 represses the genes responsible for active virus replication; dissociation of the
80 F55-RadA complex upon DNA damage leads to transcriptional derepression and
81 active replication of SSV1 (17). However, whether such a mechanism is conserved in
82 other archaeal viruses, especially in viruses infecting distantly related archaea, such as
83 halophiles, remains unknown.

84 The temperate haloarchaeal virus SNJ1 isolated from *Natrinema* sp. J7-1 resides in
85 the cytoplasm as a circular plasmid called pHH205 (9, 28). A small amount of virions
86 [with a titer of about 10^6 plaque-forming unit (pfu) mL^{-1}] is released during
87 cultivation, presumably due to spontaneous induction in some cells (Supplementary

88 Fig. 1). Upon treatment of J7-1 with the DNA damaging agent mitomycin C(MMC),
89 SNJ1 is triggered to undergo the lytic life cycle and up to 10^{10} pfu mL⁻¹ of SNJ1
90 virions are produced. Released SNJ1 viruses can infect a strain cured from SNJ1,
91 named *Natrinema* sp. CJ7 (10), and forms turbid plaques on lawns of CJ7. Here we
92 investigated how SNJ1 controls its life cycle and identified its ORF4 as a critical
93 regulator.

94

95 **Results**

96 **The discovery of clear plaque mutants of SNJ1**

97 Our lab previously used SNJ1's proviral genome, plasmid pHH205, to construct a
98 series of *E. coli*-*Natrinema* shuttle vectors (29). One of these vectors, pYC-S, was
99 obtained by insertion of the *E. coli* vector pUC19-mev at the *SacI* site of the SNJ1
100 genome (Supplementary Fig. 2). Since pYC-S contains the whole genome of SNJ1,
101 we tested whether infectious viruses could be produced from a SNJ1-cured strain
102 carrying pYC-S (CJ7/pYC-S) upon MMC treatment using a double-layer plaque assay.
103 As shown in Fig. 1a, many plaques formed on the lawn of CJ7 using supernatants
104 from MMC treated CJ7/pYC-S culture, suggesting that SNJ1 progeny viruses were
105 generated. However, unlike the homogenous turbid plaques formed by wild type SNJ1
106 viruses, viruses produced using pYC-S formed two kinds of plaques, one was similar
107 to the turbid plaques formed by wild type SNJ1, while the other was much bigger and
108 clearer (Fig. 1a). This suggested that viruses produced using pYC-S were a mixture of

109 viruses. Moreover, viruses purified from the turbid or clear plaques maintained their
110 plaque morphotypes on the lawns of CJ7 (Fig. 1a bottom). In liquid culture of CJ7,
111 addition of the turbid-plaque forming viruses slowed down the growth of CJ7
112 similarly to wild type SNJ1, while clear-plaque forming viruses completely blocked
113 its growth (Fig. 1b). Consistent with this, we were unable to obtain stable lysogen of
114 the clear-plaque SNJ1 mutant viruses, whereas lysogens of the turbid-plaque SNJ1
115 mutant viruses were obtained at the same frequency as wild type SNJ1, suggesting
116 that the clear-plaque SNJ1 mutant viruses could only undergo the lytic pathway. Since
117 disruption of the lysis-lysogeny switch of temperate viruses frequently resulted in the
118 appearance of clear plaques, we suspected that insertion of the pUC19-mev fragment
119 at the *SacI* site of SNJ1 disrupted the lysis-lysogeny life cycle of SNJ1.

120 **ORF4 is critical for the lysis-lysogeny switch of SNJ1**

121 To determine how insertion of foreign DNA disrupted the life cycle of SNJ1, we first
122 used PCR to check the genomic DNA (gDNA) integrity of the mutant viruses
123 generated from pYC-S. Interestingly, we found that a primer pair designed to amplify
124 the pUC19-mev region of pYC-S always generated products smaller than its expected
125 size using mutant SNJ1 viruses as templates. This suggested that parts of the
126 pUC19-mev fragment were lost in the SNJ1 mutant viruses. To confirm this, the
127 pUC19-mev region and its flanking sequences from 20 clear or turbid-plaque viruses
128 were amplified by PCR (HJ-test-F/R primers listed in Supplementary Table 2) and
129 sequenced. As shown in Fig. 2, all of the sequenced viral gDNA had varying degrees
130 of deletion of the pUC19-mev fragment; some also contained deletions in the flanking

131 sequences from SNJ1. The total length of pYC-S (21,425 bp) was 30% longer than
132 the length of wild type SNJ1 genome (16,492 bp, Supplementary Fig. 2) and it had
133 been reported that phage packaging systems never pack exceed about 10% over the
134 parental gDNA (30, 31). Hence, it was unsurprising that all the gDNA of mutant
135 viruses generated from pYC-S contained deletions. Examination of the length of the
136 mutant viruses genomes showed that they ranged from 16,010 bp to 18,054 bp (97%
137 to 109% of the genome length), suggesting that the capsid of SNJ1 could tolerate a
138 maximum of approximately 1.5 kb of foreign DNA.

139 Strikingly, the *orf4* gene of SNJ1 (nucleotide sequence 499-293) was disrupted in all
140 clear-plaque SNJ1 viral genomes, whereas it was intact in every turbid-plaque mutant
141 virus (Fig. 2), suggesting that disruption of *orf4* resulted in the appearance of clear
142 plaque forming viruses. To test this, the plaque morphologies of ORF4-disrupted
143 viruses were determined using viruses induced from CJ7/pYC-S-4M (start codon
144 mutation of *orf4*, SNJ1^{*orf4* mut}) and CJ7/pYC-S Δ 1-575 (deletion mutation, SNJ1 ^{Δ *orf4*}).
145 As shown in Fig. 3a, SNJ1^{*orf4* mut} and SNJ1 ^{Δ *orf4*} viruses formed only clear plaques on
146 lawns of CJ7, suggesting that ORF4 controlled the lysis-lysogeny switch of SNJ1. To
147 further test this, we checked whether expression of ORF4 in trans would restore turbid
148 plaque formation of the clear-plaque SNJ1 mutant viruses. For this test, we used a
149 plasmid containing *orf4* and its co-transcribed downstream gene *orf3* as well as
150 flanking sequences of this operon (1-656) to preserve its promoter and elements
151 critical for its expression. As shown in Fig. 3b, plaque formation of the SNJ1^{*orf4* mut}
152 and SNJ1 ^{Δ *orf4*} viruses was significantly inhibited on lawns of CJ7 expressing *orf4*

153 (with plasmid pFJ6-1-656), although some turbid plaques could form at the 10^0 to 10^{-3}
154 dilutions. On lawns of CJ7 containing the empty vector, SNJ1^{orf4 mut} and SNJ1^{Δorf4}
155 viruses formed clear plaques up to the 10^{-7} dilutions. Notably, the complementation of
156 ORF4 in CJ7 also blocked the plaque formation of SNJ1 completely. These results
157 demonstrated that ORF4 was critical for the lysis-lysogeny switch of SNJ1 and
158 suggested that it also mediated superinfection immunity of the temperate virus (see
159 below).

160 We previously found that inactivation of ORF4 affected the stability and copy number
161 of SNJ1 based *E. coli-Natrinema* shuttle vectors (29), suggesting that it was likely
162 also critical for the maintenance of the SNJ1 lysogenic state. Consistent with previous
163 observations, we found that in the presence of ORF4, the shuttle vector pYC-SHS,
164 which contained the replication and regulatory elements of SNJ1, was stably
165 maintained at about 2 copies per chromosome. However, in the absence of ORF4
166 (pYC-SHS-4M), the copy number of this plasmid increased to 20 copies per
167 chromosome and was extremely unstable (Fig. 4). Expression of ORF4 in trans
168 (pFJ6-1-656) restored stability and decreased the copy number of pYC-SHS-4M,
169 confirming the role of ORF4 in plasmid stability and copy number control. More
170 strikingly, we found that the copy number of pYC-SHS (with *orf4*) increased from 2
171 copies to 25 copies per chromosome upon MMC treatment, whereas the copy number
172 of pYC-SHS-4M (without *orf4*) was only slightly affected by MMC (35 copies
173 comparing to 20 copies per chromosome). Since pYC-SHS contained the gene
174 clusters required for SNJ1 replication and regulation, the mechanism responsible for

175 the induction of SNJ1 during MMC treatment of J7-1 was likely the same. Thus, these
176 results suggested that ORF4 acted as a repressor of replication of SNJ1 to maintain it
177 in the lysogenic state. Since the lack of ORF4 and MMC induction both lead to
178 activation of SNJ1 lytic cycle, we speculated that ORF4 may be inactivated upon
179 DNA damage, resulting in the active replication of SNJ1.

180 **ORF4 mediates superinfection immunity of SNJ1**

181 The above finding (Fig. 3b) that the presence of ORF4 in CJ7 cells inhibited plaque
182 formation of SNJ1 viruses suggested that SNJ1 had immunity against a second
183 infection by itself and ORF4 played a critical role. To confirm this, we infected CJ7 or
184 strain J7-1 with SNJ1 viruses. As shown in Fig. 5a, while SNJ1 formed plaques on
185 CJ7, J7-1 was completely immune to the infection of SNJ1, confirming that SNJ1
186 establishes superinfection immunity. Moreover, the presence of a plasmid carrying the
187 *orf3-4* operon in CJ7 completely blocked infection by SNJ1 and inactivation of ORF4,
188 but not ORF3, by a frame shift mutation abolished the ability to block SNJ1 infection.
189 This confirmed ORF4 is the critical regulator of superinfection immunity.

190 *orf4* was predicted to encode a 7.7 kDa protein possessing 68 residues. Western blot
191 of a GFP fusion of ORF4 confirmed that it encoded for a protein, although the fusion
192 protein was not functional in superinfection immunity (Supplementary Fig. 3).
193 Bioinformatic analysis of ORF4 revealed that it belonged to the MazE antitoxin
194 superfamily and showed homology with the *Bacillus subtilis* sporulation regulatory
195 protein SpoVT and the C68 protein of the hybrid virus-plasmid pSSVx (Fig. 5b).

196 These proteins usually form dimers through a swapped hairpin domain and act as
197 transcriptional regulators, which is a distinctive structural characteristic of this family
198 (32-35). Protein secondary-structure prediction suggested that ORF4 contained 5
199 β -strands and one α -helix. To determine which part of ORF4 was important for
200 superinfection immunity, it was truncated and expressed under the promoter of heat
201 shock protein 70 from *Haloferax volcanii* D52 in plasmid pFJ6. Remarkably, a
202 fragment containing only the N-terminal 33 amino acids was sufficient to block SNJ1
203 infection (Fig. 5c). Further truncation of the 33 amino acids abolished its ability to
204 restrict SNJ1 infection, demonstrating that the N-terminal 33 amino acids of ORF4
205 were necessary and sufficient for superinfection immunity.

206 **ORF4 represses DNA replication of SNJ1**

207 Generally, there are five stages in a viral life cycle: adsorption, gDNA ejection,
208 macromolecular synthesis, packaging and release. ORF4 may inhibit any step of the
209 SNJ1 viral life cycle to prevent its replication. We first checked whether adsorption of
210 SNJ1 to the host cells was blocked by ORF4. To do this, SNJ1 was incubated with
211 cells with or without SNJ1 (CJ7, J7-1, respectively) or cells with or without ORF4
212 expression (CJ7-F/pFJ6-MCS and CJ7-F/pFJ6-Hpro-*orf4*) at multiplicity of infection
213 (MOI) of 0.1, 1 and 10 for 1 h. After incubation, the titer of unabsorbed viruses was
214 quantified by double-layer plaque assay and compared to the titer of the viruses
215 before the adsorption assay. The difference of the titers before and after the adsorption
216 assay was considered as the adsorption efficiency. As shown in Fig. 6a, there was no
217 obvious difference in adsorption efficiency in the presence or in the absence of SNJ1,

218 nor with or without ORF4 expression at low or high MOI. Therefore, we concluded
219 that ORF4 did not act by preventing viral adsorption.

220 Next, we tested whether viral gDNA ejection was inhibited by ORF4. The relative
221 copy number of viral gDNA in the host cells with or without ORF4 expression were
222 quantified 1 h post infection by qPCR. The single-copy gene *radA* located on the
223 chromosome was used as a reference, while *orf14* of SNJ1 was used for viral gDNA.

224 As shown in Fig. 6b, at a MOI of 0.1, copies of viral gDNA in cells without ORF4
225 (0.0065 in CJ7, 0.0068 in CJ7/pFJ6-MCS) was about 3-fold higher than cells with
226 ORF4 (0.0020 in CJ7-F/pFJ6-Hpro-*orf4*). The difference increased to about 6-fold at
227 a MOI of 1 (0.0691, 0.0601 vs 0.0106) and 9-fold at a MOI of 10 (0.8877, 0.8100 vs
228 0.1080). These results suggested that ORF4 may prevent gDNA from ejecting into the
229 host cells. However, we could not exclude the possibility that the ejected gDNA had
230 been degraded in the presence of ORF4 given that this was examined 1 hour post
231 infection. It was also possible that ejection of gDNA was not affected, but replication
232 of the ejected gDNA was suppressed by ORF4 (see below).

233 To test whether replication of SNJ1 was repressed by ORF4, we quantified the copy
234 number of gDNA of SNJ1 in the infected cells every hour post infection for 9 h. For a
235 better comparison, we infected cells without ORF4 (CJ7 and CJ7/pFJ6-MCS) at a
236 MOI of 0.1, while cells with ORF4 (CJ7-F/pFJ6-Hpro-*orf4*) at a MOI of 0.5 to ensure
237 similar gDNA copy number of SNJ1 at 1 h post infection (0.0046, 0.0039 and 0.0038
238 copies per chromosome, respectively). As shown in Fig. 6c, in the absence of ORF4
239 (strain CJ7 and CJ7/pFJ6-MCS), the viral gDNA duplicated rapidly over time and

240 reached up to 2.5280 or 2.0998 copies per chromosome 9 h post infection. However,
241 the viral gDNA was barely replicated in the strain CJ7-F/pFJ6-Hpro-*orf4* (0.0582
242 copies per chromosome). These results showed that gDNA replication in the host cell
243 was significantly repressed in the presence of ORF4.

244 To ensure that ORF4 repressed replication of the SNJ1 gDNA instead of degrading it,
245 we repeated the experiment by southern blot analysis using probes that specifically
246 recognized SNJ1. Note that restriction digestion of the gDNA was not carried out in
247 this assay to preserve the integrity of the sample and as a consequence multiple bands
248 of SNJ1 gDNA could show up as the DNA molecule could be supercoiled. Consistent
249 with the above results, gDNA bands were detected in strain CJ7 and CJ7/pFJ6-MCS 1
250 h post infection and accumulated extensively as time increased in the absence of
251 ORF4 (Fig. 6d). However, only a weak signal of SNJ1 genome was observed in the
252 ORF4 expressing strain CJ7-F/pFJ6-Hpro-*orf4* even 9 h post infection. More
253 importantly, SNJ1 genome remained intact during the whole cultivation process,
254 suggesting that ORF4 did not prevent replication by degrading viral DNA. In
255 conclusion, these results suggested that ORF4 precluded SNJ1 infection through
256 inhibition of viral gDNA replication.

257 **ORF4 is highly conserved in SNJ1-like proviruses**

258 Because ORF4 played such an important role in the SNJ1 life cycle, its presence in a
259 genome may indicate the existence of a SNJ1-like provirus. Thus, we searched for
260 ORF4-like proteins using a protein-protein BLAST algorithm. We found that an ORF

261 in *Natrinema versiforme* strain BOL5-4 plasmid pNVE19 and an ORF in
262 *Haloterrigena jeotgali* strain A29 plasmid unnamed5 shared high homologies to
263 ORF4 (94% and 83% identity, respectively). When the genomes of these two
264 plasmids were aligned with the genome of SNJ1, we found that they were highly
265 similar, indicating that they could be SNJ1-like proviruses (Fig. 7). First, their
266 genomes were about the same size and encoded roughly similar number of ORFs.
267 BOL5-4 plasmid pNVE19 was 18,925 bp and contained 28 putative genes (GenBank
268 Acc. No. NZ_CP040333), while A29 plasmid unnamed5 was 17,189 bp and contained
269 31 putative genes (GenBank Acc. No. CP031302)(36). Second, most of the ORFs on
270 these two plasmids were homologous to ORFs of SNJ1 and arranged into clusters
271 similarly as SNJ1. Most importantly, genes homologous to the ORFs important for
272 SNJ1 replication shared identity over 40% and homologs of the core structural
273 elements for *Sphaerolipoviridae* were also present in these two plasmids (37). These
274 included homologs of the two major capsid proteins and the putative packaging
275 ATPase of SNJ1 (colored blue and pink, respectively). Based on these features, these
276 two plasmids were likely SNJ1-like proviruses and ORF4-like protein may play a
277 critical role in the regulation of their life cycles.

278

279 Discussion

280 Overall, our results showed that ORF4 is a master regulator of the life cycle of SNJ1
281 (Fig. 8). It not only regulates the lysis-lysogeny switch of SNJ1, but also mediates
282 superinfection immunity, two phenomena which are presumably widely present but

283 have rarely been studied for archaeal viruses. Sequence analysis of ORF4 showed that
284 it contains the SpoVT/AbrB-like domain and shares homology with proteins
285 belonging to the antitoxin MazE superfamily. Proteins of the SpoVT/AbrB family
286 normally act as transcriptional regulators (38-40). MazE is the antidote to the toxin
287 MazF, and MazE-MazF in *E. coli* is a regulated prokaryotic chromosomal addiction
288 module. MazE is a labile protein that is degraded by ClpAP serine protease (41, 42).
289 We suspect that ORF4 works as a transcriptional repressor of the genes responsible
290 for the lytic pathway.

291 The expression level of ORF4 in the host cell appears to be critical for determining
292 the route of the SNJ1 infection. In the absence of ORF4, SNJ1 formed clear plaques,
293 whereas in its presence, SNJ1 formed turbid plaques, and with its ectopic expression,
294 SNJ1 infection was blocked. This suggests that the lysis-lysogeny switch is controlled
295 by a ratio of ORF4 with a yet to be discovered SNJ1-encoded protein(s). The
296 observations that MMC treatment and disruption of ORF4 both resulted in the active
297 replication of SNJ1 and increased the copy number of SNJ1-based plasmids indicate
298 that DNA damage in the host cell may lead to the inactivation of ORF4. Presumably,
299 ORF4 is cleaved and/or degraded by host proteases in a manner similar to the
300 cleavage/degradation of the cI repressor of λ phage (23). It is also possible that ORF4
301 dissociates from its target sites like the F55 repressor of SSV1 (43). Future studies to
302 test the ability of ORF4 to bind DNA and regulate gene expression as well as its
303 inactivation upon DNA damage in the host cell will undoubtedly provide critical
304 missing information on the mechanism of the lysis-lysogeny switch of SNJ1.

305 Superinfection immunity has been well documented for many bacteriophages and
306 eukaryotic viruses (44-46). However, only a couple of cases have been reported for
307 archaeal viruses. It was found that the adsorption of *Sulfolobus islandicus* rod-shaped
308 virus 2 (SIRV2) to its host cells was significantly reduced by a previous infection,
309 indicating that SIRV2 established superinfection exclusion (47). However, the exact
310 mechanism underlying this phenomenon has not yet been elucidated. In addition,
311 hyperthermophilic archaeal viruses SPV1 and SPV2 carry mini-CRISPR arrays
312 containing spacers against each other and, consistently, seem to restrict each others
313 replication. Thus, it has been suggested that virus-borne mini-CRISPR arrays might
314 represents a distinct mechanism of heterotypic superinfection exclusion (48). Here we
315 present evidences that lysogenic SNJ1 confers immunity against subsequent
316 infections and identify ORF4 as a critical factor in this process by repressing SNJ1
317 genome replication. However, we cannot exclude the possibility that it may also block
318 gDNA ejection into the host cells because our results showed that at early stage of
319 infection (1 h post infection) gDNA in cells without ORF4 were remarkably higher
320 than cells with ORF4. Strikingly, we found that only the N-terminal 33 amino acids of
321 ORF4 are necessary and sufficient for blocking SNJ1 infection. It is likely that the
322 N-terminal 33 amino acids of ORF4 is sufficient to bind DNA and repress
323 transcription of the lytic pathway genes. However, biochemical characterization of
324 ORF4 is necessary to confirm this and reveal its mechanism of action.

325 In conclusion, we showed that the temperate haloarchaeal virus SNJ1 employs a
326 lysis-lysogeny switch and establishes superinfection immunity. We also identified

327 ORF4 as a key factor for both of these processes and found that it acts as a repressor
328 of SNJ1 replication. We suggest that SNJ1 could be used as a model system to
329 understand the regulation of archaeal viral life cycle and virus-host interactions. Using
330 the SNJ1 mutant viruses and *E. coli-Natrinema* shuttle vectors generated in this study,
331 many other viral proteins of SNJ1 and host factors necessary for the regulation of
332 SNJ1 life cycle could be discovered. Characterization and elucidation of how these
333 proteins and ORF4 work should greatly increase our understanding of the enigmatic
334 archaeal virosphere.

335

336 **Materials and Methods**

337 **Strains, culture conditions and transformation methods**

338 All strains, plasmids and primers used in this study were listed in Supplementary
339 Table 1-3. Different derivatives of *Narinema* sp. J7 were cultured in Halo-2 medium
340 at 45°C as described before (10). Halo-2 medium contained 250 g NaCl, 30 g
341 $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 2.5 g Lactalbumin hydrolysate (Difco Laboratories) and 2 g Bactoyeast
342 extract (Difco Laboratories) per liter of water. Casamino Acids medium (Hv-Ca) or 18%
343 modified growth medium (MGM) were prepared as described before (29, 49). Hv-Ca
344 contained 144 g NaCl, 18 g $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 21 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 4.2 g KCl, 5 g Amicase
345 (Sigma), 0.5 g CaCl_2 and 30 mL 1M Tris-HCl (pH = 7.5) per liter of water. 18%
346 MGM contained 144 g NaCl, 18 g $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 21 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 4.2 g KCl, 5 g
347 Peptone (Difco Laboratories), 3 g Bacto yeast extract (Difco Laboratories), 0.5 g
348 CaCl_2 and 30 mL 1M Tris-HCl (pH = 7.5) per liter of water.

349 Transformation of *Narinema* sp. CJ7 and CJ7-F was performed using the modified
350 polyethylene glycol method as described previously (50, 51). CJ7 transformed with
351 pYC vectors were plated on 18% MGM plates with 5 $\mu\text{g mL}^{-1}$ mevinolin (Mev),
352 while CJ7-F transformed with pFJ6 vectors were selected on Hv-Ca medium. *E. coli*
353 were cultured in Luria-Bertani medium at 37°C, supplemented with ampicillin (0.1
354 mg mL⁻¹) when necessary. *E. coli* were transformed following the CaCl₂ method (52).
355 Solid and soft agar media were prepared by adding 12 g L⁻¹ and 5 g L⁻¹ agar
356 respectively.

357 **Plasmid construction**

358 To construct pYC-S-4M, overlap extension PCR was performed by primer pairs
359 pYC-S-*Afl*II-F/4M-R and 4M-F/pYC-S-*Nco*I-R using pYC-S as a template. PCR
360 products were ligated into *Afl*II-*Nco*I digested pYC-S by Hieff Clone™ Plus One Step
361 Cloning Kit (Yeasen, China). To construct pYC-S Δ 1-575, the *Sac*I-*Nco*I fragment on
362 the pYC-S was replaced with the *Sac*I-*Nco*I fragment on the pYC-1 by T4 DNA
363 Ligase (Takara). To construct *E. coli*-*Narinema* sp. J7 shuttle vectors containing
364 full-length SNJ1, SNJ1 proviral genomes pHH205 was extracted from strain J7-1
365 using TIANpure Mini Plasmid Kit (TIANGEN). Purified pHH205 was digested with
366 FastDigest *Sac*I restriction enzyme (ThermoFisher Scientific) and ligated with *Sac*I
367 digested pUC-mev.

368 Derivatives of pFJ6 plasmids were all constructed by the same approach. For
369 pFJ6-1-656, a DNA fragment containing nucleotide 1-656 of SNJ1 viral genomewas

370 amplified with primer pairs *Sph*I-1-F/*Mun*I-656-ORF4-R from J7-1. The product was
 371 digested with *Mun*I-*Sph*I and ligated into *Mun*I-*Sph*I digested pFJ6-MCS by T4 DNA
 372 Ligase (Takara). Frame-shift mutations were introduced by overlap extension PCR
 373 using primer pairs *Sph*I-1-F/(ORF3-FSM-R, ORF4 FSM-R) and (ORF3-FSM-F,
 374 ORF4 FSM-F)/*Mun*I-656-ORF4-R. For pFJ6-Hpro, target genes were amplified by
 375 *Mun*I-Hpro-F/*Not*I-Hpro-R from pYCJ-H , the product was digested with *Mun*I-*Not*I
 376 and ligated into pFJ6-MCS. For pFJ6-Hpro-*orf4*, pFJ6-Hpro-*orf4*-4FSM and
 377 pFJ6-Hpro-*orf4*^x, target genes were amplified by
 378 *Not*I-ORF4-499-F/(*Sph*I-ORF4-293-R, *Sph*I-ORF4-150-R, *Sph*I-ORF4-99-R,
 379 *Sph*I-ORF4-72-R, *Sph*I-ORF4-48-R, *Sph*I-ORF4-24-R) and (*Not*I-ORF4-FSM-F,
 380 *Not*I-ORF4-400-F)/*Sph*I-ORF4-293-R respectively from J7-1, then the products were
 381 digested with *Not*I-*Sph*I and ligated into pFJ6-Hpro.

382 For pFJ6-PphaRP-GFP, PCR was performed by primer pairs
 383 ORF4-GFP-1-F/ORF4-GFP-3-R using plasmid pRF as a template(53). PCR products
 384 were ligated into *Afl*II-*Sph*I digested pFJ6-MCS by Hieff Clone™ Plus One Step
 385 Cloning Kit (Yeasen, China). For pFJ6-PphaRP-ORF4-GFP-1 and
 386 pFJ6-PphaRP-ORF4-GFP-2, target genes were amplified by primer pairs
 387 ORF4-GFP-1-F/ORF4-GFP-1-R, ORF4-GFP-2-F/(ORF4-GFP-2-R, ORF4-GFP-4-R)
 388 and (ORF4-GFP-3-F, ORF4-GFP-4-F)/ORF4-GFP-3-R respectively, and PCR
 389 products were ligated into *Afl*II-*Sph*I digested pFJ6-MCS as well.

390 All of the plasmids were transformed into *E. coli* DH5α and JM110 successively to
 391 get demethylated plasmids. Finally, the plasmids were transformed into *Natrinema* sp.

392 CJ7 or CJ7-F accordingly.

393 **SNJ1 induction, propagation and infection procedures**

394 SNJ1 was induced from J7-1 strain by MMC (Roche). J7-1 was cultivated in Halo-2
395 medium at 45°C for about 24 h to late-exponential phase ($OD_{600} \approx 0.6$), and treated
396 with MMC ($1 \mu\text{g mL}^{-1}$) for 30 min at 37°C with aeration. Cells were collected by
397 centrifugation (10000 rpm, 5 min) and resuspended in the same volume of Halo-2
398 medium. After 24 h of cultivation at 45°C, 200 rpm, the culture was centrifuged at
399 10000 rpm for 20 min, and the supernatant was collected and passed through a filter
400 ($0.22 \mu\text{m}$) to remove cell debris. The titer of the virus stock was measured by
401 double-layer plaque assay as follows.

402 SNJ1 titers were calculated as pfu mL^{-1} as described previously(54), with minor
403 modifications. 100 μL of SNJ1 virus stock dilutions and 400 μL of CJ7 culture
404 (late-exponential phase, $OD_{600} \approx 0.6$) were mixed with 4 mL of soft melted Halo-2
405 medium (top layer), then poured onto Halo-2 solid plates (bottom layer) immediately.
406 The plates were incubated at 37°C for 48 h and counted. If necessary, CJ7 cells at
407 early-exponential growth phase ($OD_{600} \approx 0.3$) were collected and infected with SNJ1
408 ($\text{MOI} = 10$) to promote virus titer.

409 The mutant virus stocks were obtained from CJ7/pYC-S, CJ7/pYC-S-4M and
410 CJ7/pYC-S $\Delta 1$ -575 by MMC treated as same as above. Late exponential phase culture
411 of CJ7 was infected with mutant viruses by double-layer plaque assay, then the plates
412 were incubated at 37°C for 48 h and photographed.

413 Analysis of the lytic ability of different viruses to CJ7

414 CJ7 was cultured in Halo-2 medium at 45°C to early-exponential growth phase
415 ($OD_{600} \approx 0.2$). The same amount of viruses (SNJ1, two turbid and clear-plaque viruses
416 from CJ7/pYC-S culture) was added into CJ7 culture and incubated at 45°C for an
417 hour. Cells were collected by centrifugation (10000 rpm, 5 min) and resuspended in
418 the same volume of Halo-2 medium. The cultures were then cultivated at 45°C, 200
419 rpm and the growth curve was monitored for 49 h post infection by measuring OD_{600} .
420 CJ7 without virus infection was set as a control.

421 Superinfection immunity assay

422 Superinfection immunity assay was performed by the double-layer plaque assay with
423 minor modifications. First, 400 μ L of late-exponential phase cultures of indicated
424 strains were mixed with soft melted Halo-2 agar (4 mL) and poured onto Halo-2 agar
425 plates immediately. 10 minutes later, when the soft agar was solidified, 2 μ L of the
426 ten-fold serial dilutions of SNJ1 virus was spotted onto the plates carefully. The plates
427 were incubated at 37°C for 48 h and photographed.

428 Western blot assay

429 CJ7/pFJ6-PphaRP-GFP and CJ7/pFJ6-PphaRP-ORF4-GFP were cultivated in Halo-2
430 medium to late-exponential phase and crude extracts were taken and loaded for
431 SDS-PAGE. Anti-GFP antibodies (TransGen Biotech) were used for Western blot
432 assay of GFP and ORF4-GFP fusion protein.

433 Adsorption assay

434 SNJ1 was incubated with 20 mL of early-exponential phase ($OD_{600} \approx 0.3$) of cultures
435 indicated strains at different MOI (0.1, 1 and 10) for about 1 h at 45°C. After
436 absorption, the culture was centrifuged (10000 rpm, 5 min), supernatant was collected
437 and titer of unbound viruses in supernatant was measured by double-layer method.
438 Adsorption efficiency was determined by comparing the titer before and after virus
439 adsorption. Three independent experiments were performed, and error bars indicated
440 standard deviations.

441 **Quantification of viral gDNA**

442 The relative copy number of viral gDNA intercellular 1 h post infection was detected
443 by qPCR method using cells collected from adsorption assay as templates. The
444 templates from different cultures were prepared as described previously (29, 55).
445 Briefly, 1 mL aliquot of the cell culture was harvested by centrifugation, and washed
446 twice by Halo-2 medium to eliminate the free viruses in supernatant. Cells were
447 resuspended in 1 mL of 18% (wt/vol) NaCl solution. To avoid the negative effect of
448 high salt concentration on qPCR, 10 μ L of the cell suspension was added to 490 μ L of
449 distilled water, which also resulted in rapid cell lysis. A single-copy gene *radA* located
450 on the chromosome of host strain was used as a reference, while *orf14* of SNJ1 genome
451 was used as target gene. The specific primers used for qPCR were listed in
452 Supplementary Table 3. For the reactions, 20 μ L mixtures were prepared containing 5
453 μ L of template, 10 μ L of iTaq Universal SYBR Green Supermix (Bio-Rad, USA), 1
454 μ L of primer pairs (10 μ M) and 4 μ L of distilled water. Amplification was performed
455 according to the manufacturer's instructions. Finally, the qPCR data were analyzed by

456 $2^{-\Delta C_T}$ method (56). Three independent experiments were performed, and error bars
457 indicated standard deviations.

458 **SNJ1 replication assay**

459 SNJ1 was incubated with 100 mL of early-exponential phase ($OD_{600} \approx 0.3$) cultures of
460 CJ7 or CJ7-F/pFJ6-MCS at a MOI of 0.1 and CJ7-F/pFJ6-Hpro-*orf4* at a MOI of 0.5
461 for 1 h at 45°C. After 1 h of absorption, the culture was centrifuged (10000 rpm, 20
462 min) to end the infection procedure. Cell pellets were washed twice with fresh Halo-2
463 medium to remove the free viruses, resuspended in the same volume of Halo-2
464 medium, and incubated at 45°C for 9 h. Samples were taken every hour post infection.
465 qPCR analyses were performed using the same primer pairs and procedure as in
466 **Quantification of viral gDNA**. The qPCR data were analyzed by $2^{-\Delta C_T}$ method (56)

467 **Genomic integrity of SNJ1 by Southern blot**

468 CJ7, CJ7-F/pFJ6-MCS and CJ7-F/pFJ6-Hpro-*orf4* cells were infected by SNJ1 as
469 described in **SNJ1 replication assay** except that SNJ1 was incubated at a MOI of 5
470 with all strains. Multiple aliquots of 8 mL of culture were collected every two hours
471 after 1 h of infection. To remove the unbound viruses, cells were collected by
472 centrifugation (10000 rpm, 5 min) and washed twice by the same volume of Halo-2
473 medium. The total genomic DNA of infected cells was extracted according to the
474 online protocol (<http://www.haloarchaea.com/resources/halohandbook>) with
475 modifications. Briefly, cell pellets were resuspended gently in 2 mL of distilled water
476 to completely lyse the cells. 2 mL of phenol-chloroform solution was mixed the cell

477 lysate to extract proteins (and probably some carbohydrate). Finally, the genomic
478 DNA was precipitated by adding 2 volumes of ethanol and dissolved in 150 µl of TE
479 buffer. 5 µL of DNA preparations were electrophoresed on 0.8 % agarose gel. The
480 DNA bands were then transferred onto positively charged nylon membranes with
481 alkaline transfer buffer according to the online protocol (Molecular Cloning chapter 6
482 protocol 8, <http://www.molecularcloning.com>). The primers ORF7-F and ORF11-R
483 were designed to synthesize a probe corresponding to the region from 1,483 to 2,537
484 bp of the SNJ1 genome (Supplementary Table 3). Southern blot was performed as
485 described before (57).

486 The DNA probe preparation, hybridization and detection were performed using DIG
487 High Prime DNA Labeling and Detection Starter kit I (Roche) according to the
488 manufacturer's instructions.

489 **Determination of plasmid copy number and stability**

490 CJ7/pYC-SHS and CJ7/pYC-SHS-4M were cultured to late-exponential phase (24 h)
491 in 18% MGM + Mev medium and treated with MMC (1 µg mL⁻¹) for 30 min, while
492 cultures without MMC were set as controls. Cells were collected by centrifugation
493 and washed twice in same volume of 18% MGM to remove MMC. Cell pellets were
494 resuspended in 18% MGM + Mev medium and cultured for 24 h. Samples were taken
495 for qPCR analysis using primer pairs vector-F/vector-R, orf14-F/orf14-R and
496 radA-F/radA-R to determine the relative plasmid copy number to chromosome. The
497 qPCR data were analyzed by 2^{-ΔC_T} method (56). Three independent experiments were

498 performed, and error bars indicated standard deviations.

499 For plasmid stability assay of pYC-SHS and pYC-SHS-4M during passage: 100 μ L of
500 stationary phase culture of CJ7/pYC-SHS and CJ7/pYC-SHS-4M in Halo-2 medium
501 was inoculated into 5 mL of Halo-2 medium every day. Samples were taken and
502 measured by qPCR using primer pairs vector-F/vector-R, orf14-F/orf14-R and
503 radA-F/radA-R. Three independent experiments were performed, and error bars
504 indicated standard deviations. The qPCR data were analyzed by $2^{-\Delta C_T}$ method (56)

505 **Bioinformatic and statistical analysis.**

506 Homologous proteins were searched by using BlastP and SyntTax (58), and conserved
507 domains were detected by CD-search at National Center for Biotechnology
508 Information (NCBI). Clustal Omega and Constraint-based Multiple Alignment Tool at
509 NCBI (COBALT) was used for multiple protein alignments
510 (<https://www.ebi.ac.uk/Tools/msa/clustalo>,
511 <https://www.ncbi.nlm.nih.gov/tools/cobalt/>). The secondary-structure of ORF4 was
512 predicted by PSIPRED in MPI Bioinformatics Toolkit
513 (<https://toolkit.tuebingen.mpg.de>) (59, 60). The global alignment of two proteins were
514 performed by EMBOSS Needle tool at EMBI-EBI
515 (https://www.ebi.ac.uk/Tools/psa/emboss_needle/).

516

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685 **Author Contributions**

686 B.B.C, X.D.C and S.D. designed the research; B.B.C, Z.C, Y.C.W, H.G, L.S.S, J.W,
687 S.S.O and W.Q.G performed the research; B.B.C, M.K., X.D.C and S.D analyzed data
688 and wrote the manuscript.

689 **Competing interests**

690 The authors declare no competing interests.

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695 **Figure legends**

696 **Fig 1. Discovery of clear-plaque mutants of SNJ1. a.** Viruses obtained from
697 CJ7/pYC-S culture formed both clear and turbid plaques on lawns of CJ7. Late
698 exponential phase culture of CJ7 was infected with SNJ1 viruses or viruses generated
699 using CJ7/pYC-S. SNJ1 formed turbid plaques, while viruses generated using pYC-S
700 formed two kinds of plaques. Arrows represented turbid plaques and triangles

701 represented clear plaques. Viruses purified from the turbid or clear plaques were
702 propagated and used to infect CJ7 as above, the morphotypes of the plaques were
703 maintained for either virus. **b.** The ability of different viruses to lyse cells. The same
704 amount of viruses was added into CJ7 culture ($OD_{600} \approx 0.1$). Growth of the culture
705 was monitored for 49 h post infection by measuring OD_{600} . SNJ1-Ca/Cb and
706 SNJ1-Ta/Tb represented two clear-plaque viruses and two turbid-plaque viruses
707 isolated from lawns of CJ7 infected with viruses from CJ7/pYC-S culture,
708 respectively. CJ7 without virus infection was used as a control. The experiment was
709 repeated twice, and error bars indicated standard deviations.

710

711 **Fig 2. Schematic diagrams of the genomic deletion of 20 turbid and clear plaque**
712 **viral genomes. a.** Scheme of SNJ1 genome from 1 bp to 1,134 bp and locations of
713 genomic deletion of turbid/clear plaque viruses of SNJ1. The putative ORFs of SNJ1
714 were indicated by numbered arrows, while the end site of genomic deletion in
715 turbid/clear plaque viruses were marked by T1-T20 and C1-C20. Red dotted arrow
716 represented the region of genomic deletion. Detailed deletion location was shown in **b**
717 (turbid plaque viruses) and **c** (clear plaque viruses). The pUC19-mev fragment and
718 flanking sequences of 20 turbid and clear plaque viruses were amplified by HJ-F/R
719 primer pair and sequenced. Black solid lines represented genomes packaged in viral
720 particles, while black dotted lines represented deletions. The putative ORFs were
721 indicated by numbered arrows and the *SacI* site, where foreigner DNA was inserted,
722 was indicated. The numbers on the right side represented the start (x) and end (y)

723 location of the genomic deletion. (z) represented the genome size of mutant SNJ1
724 viruses and the smaller genomes were noted in bold, compared to the wild-type SNJ1
725 (16,492 bp).

726

727 **Fig 3. Identification of *orf4* as a critical factor for SNJ1 lysis-lysogeny switch. a.**

728 *orf4* disrupted viruses formed only clear plaques on lawns of CJ7. Late exponential
729 phase culture of CJ7 was infected with SNJ1^{*orf4* mut} and SNJ1 ^{Δ *orf4*} viruses (generated
730 from CJ7/pYC-S-4M and CJ7/pYC-S Δ 1-575 culture respectively), and only clear
731 plaques were observed. **b.** Expression of ORF4 in trans restored turbid plaque
732 formation of clear-plaque SNJ1 mutant viruses and inhibited plaque formation
733 dramatically. Ten-fold serial dilutions of SNJ1 viruses or its clear plaque mutants were
734 spotted onto the lawns of CJ7-F/pFJ6-MCS, or CJ7-F/pFJ6-1-656 (with *orf4*). Plates
735 were incubated at 37°C for 48 h and photographed. The maximum dilution for
736 observed plaques was highlighted by asterisk.

737

738 **Fig 4. ORF4 is critical for stability and copy number control of SNJ1-based**

739 **plasmids. a.** The relative copy number of SNJ1-based plasmid pYC-SHS (with *orf4*)
740 and pYC-SHS-4M (without *orf4*) in CJ7 cells with or without MMC treatment. WT
741 represents wild type pYC-SHS plasmid, Δ *orf4* represents pYC-SHS-4M (start codon
742 mutation of *orf4*) and Δ *orf4*+*orf4* represents pFJ6-1-656 complemented
743 pYC-SHS-4M. These plasmids were transformed to CJ7 strain and cultured to
744 late-exponential phase (24 h) in 18% MGM + Mev medium and treated with MMC (1

745 $\mu\text{g mL}^{-1}$) for 30 min, while cultures without MMC treated were set as control. Cells
746 were collected by centrifugation and washed twice in same volume of 18% MGM to
747 remove MMC. Cell pellets were resuspended in 18% MGM + Mev medium and
748 cultured for 24 h. Samples were taken for qPCR analysis using primer pairs
749 vector-F/vector-R, orf14-F/orf14-R and radA-F/radA-R to determine the relative
750 plasmid copy number to chromosome. Three independent experiments were
751 performed, and error bars indicated standard deviations. **b.** Plasmid stability of
752 pYC-SHS and pYC-SHS-4M during passage. 100 μL of stationary phase culture of
753 CJ7/pYC-SHS, CJ7/pYC-SHS-4M and CJ7/pYC-SHS-4M+pFJ6-1-656 was
754 inoculated into 5 mL of Halo-2 medium every day. Samples were taken and measured
755 by qPCR using primer pairs vector-F/vector-R, orf14-F/orf14-R and radA-F/radA-R
756 for five days. Three independent experiments were performed, and error bars
757 indicated standard deviations.

758

759 **Fig. 5. ORF4 mediates superinfection immunity of SNJ1. a.** Expression of ORF4
760 confers resistance to SNJ1 infection in CJ7. To test the immunity to against SNJ1,
761 ten-fold serial dilutions of SNJ1 virus stocks were spotted onto lawns of CJ7, J7-1 and
762 CJ7-F strains carrying pFJ6 plasmids with or without *orf3* and *orf4*. The maximum
763 dilution for observed plaques was highlighted by asterisk. **b.** Sequence alignment of
764 ORF4 with other MazE/SpoVT family members. C68 protein from hybrid
765 virus-plasmid pSSVx (residues 1-68, PDB code 3O27)(32), the N-terminal domains
766 of MazE (residues 1-53, PDB code 1UB4)(33), AbrB (residues 1-51, PDB code

767 1YSF)(34), and SpoVT (residues 1-55, PDB code 2W1T)(35) are aligned with ORF4
768 by Clustal Omega. The secondary-structure of ORF4 was predicted by PSIPRED in
769 MPI Bioinformatics Toolkit. ORF4 contains 5 β -strands (light-grey arrows) and one
770 α -helix (dark-grey cylinder). The same color is used for other proteins. **c.** The
771 N-terminal 33 amino acids of SNJ1 were necessary and sufficient for immunity to
772 against SNJ1. Ten-fold serial dilutions of SNJ1 were spotted onto lawns of CJ7-F
773 harboring plasmid pFJ6-MCS or its derivatives carrying different portion of ORF4,
774 superscript denotes the amino acid residues of ORF4. Hpro stands for the promoter of
775 heat-shock protein 70 from *Haloferax volcanii* DS52. Plates were incubated at 37°C
776 for 48 h and photographed.

777

778 **Fig. 6. ORF4 blocks SNJ1 infection by inhibiting viral gDNA replication. a.** ORF4
779 did not affect SNJ1 adsorption to host cells CJ7. SNJ1 was incubated with
780 early-exponential phase cultures of CJ7, J7-1, CJ7-F/pFJ6-MCS (- *orf4*) and
781 CJ7-F/pFJ6-Hpro-*orf4* (+ *orf4*) at different MOI (0.1, 1 and 10) for about 1 h at 45°C.
782 After absorption, the culture was centrifuged, and the titer of unbound viruses in
783 supernatant was measured by double-layer method. Adsorption efficiency was
784 determined by comparing the titer before and after virus adsorption. Three
785 independent experiments were performed, and error bars indicated standard deviations.
786 **b.** Relative copy number of viral gDNA intercellular 1 h post infection. Indicated
787 strains were infected with SNJ1 as in (a). After 1 h of adsorption, the cells were
788 centrifuged and the relative copy number of viral gDNA intercellular were measured

789 by qPCR using primer pairs orf14-F/orf14-R and radA-F/radA-R, which represented
 790 proviral genome and host chromosome, respectively. The bars represent the means
 791 and standard deviations of three independent experiments. Significance testing against
 792 CJ7-F/pFJ6-Hpro-*orf4* was performed using a one-sample t-test (***: $P < 0.001$, **: P
 793 < 0.01). **c.** ORF4 repressed SNJ1 genome replication. SNJ1 was incubated with CJ7
 794 or CJ7-F/pFJ6-MCS at a MOI of 0.1 and CJ7-F/pFJ6-Hpro-*orf4* at a MOI of 0.5 for 1
 795 h. The cells were collected and washed in fresh Halo-2 medium twice to eliminate
 796 free viruses. Cells were then cultivated for 8 h and samples were taken every hour
 797 post infection. qPCR analyses were performed using the same primer pairs as in **(b)**.
 798 The bars represent the means and standard deviations of three independent
 799 experiments. Significance testing against CJ7-F/pFJ6-Hpro-*orf4* was performed using
 800 a one-sample t-test (***: $P < 0.001$, **: $P < 0.01$, *: $P < 0.05$). **d.** Genomic integrity of
 801 provirus was not affected by ORF4. Experiments were performed as in **(c)** expect that
 802 SNJ1 was incubated at a MOI of 5 with all strains and samples for southern blot
 803 analysis were taken every two hours after 1 h of infection. The DNA samples were
 804 electrophoresed on agarose gels and transferred onto positively charged nylon
 805 membranes with alkaline transfer buffer. A specific probe recognized nucleotide
 806 1,483-2,537 bp of SNJ1 was used for southern blot analysis. The DNA marker were
 807 shown on the left. C: CJ7 strain; -: CJ7-F/pFJ6-MCS strain; +: CJ7-F/pFJ6-Hpro-*orf4*
 808 strain.

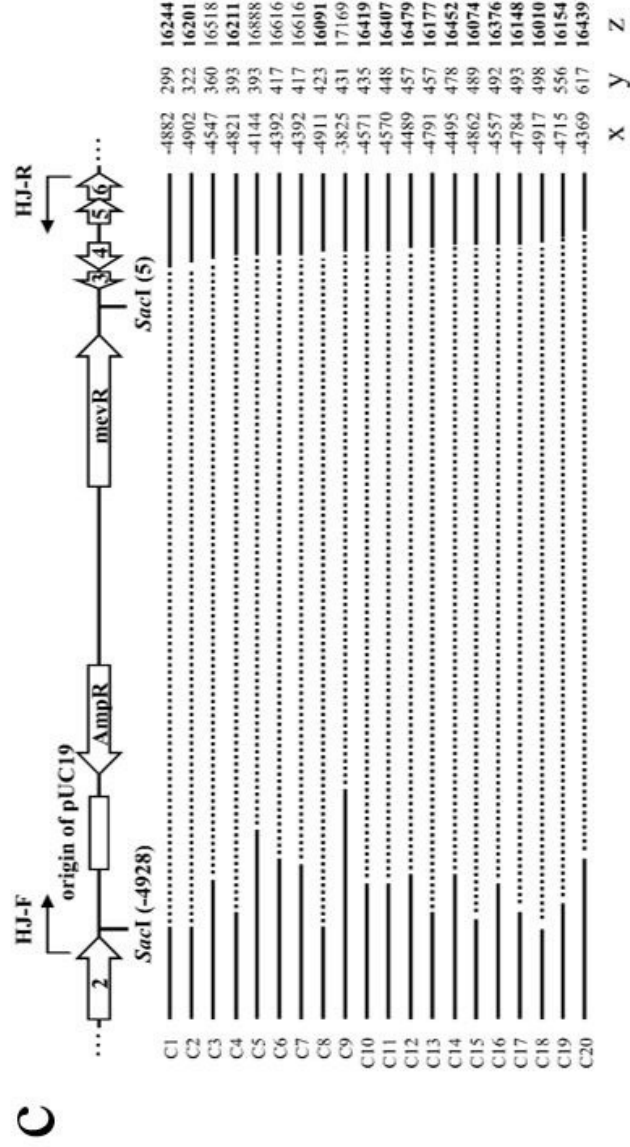
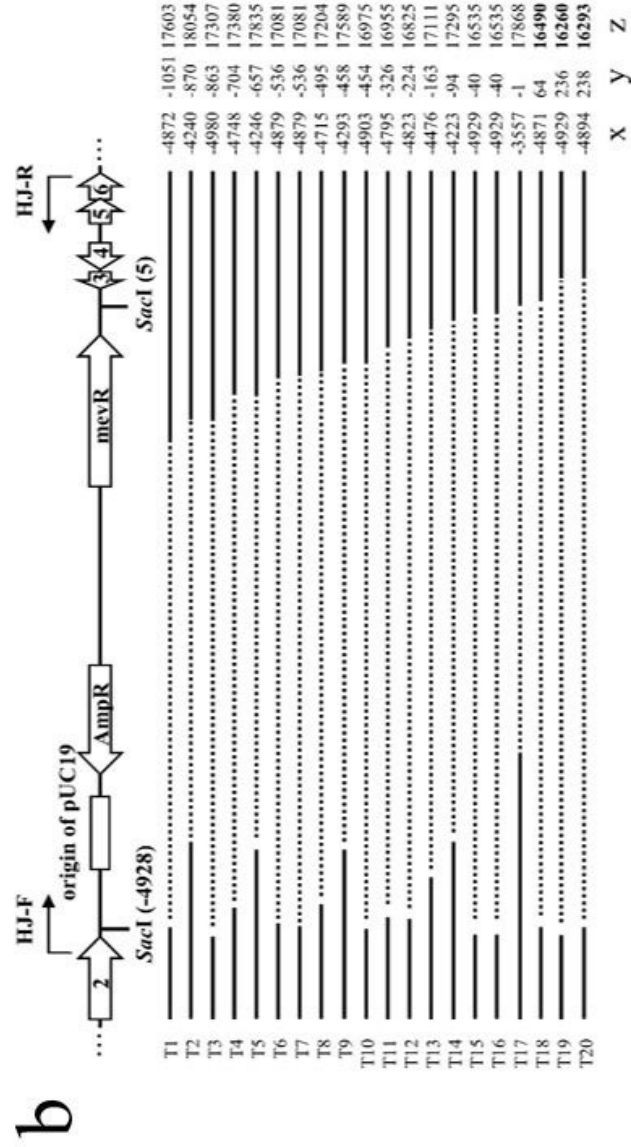
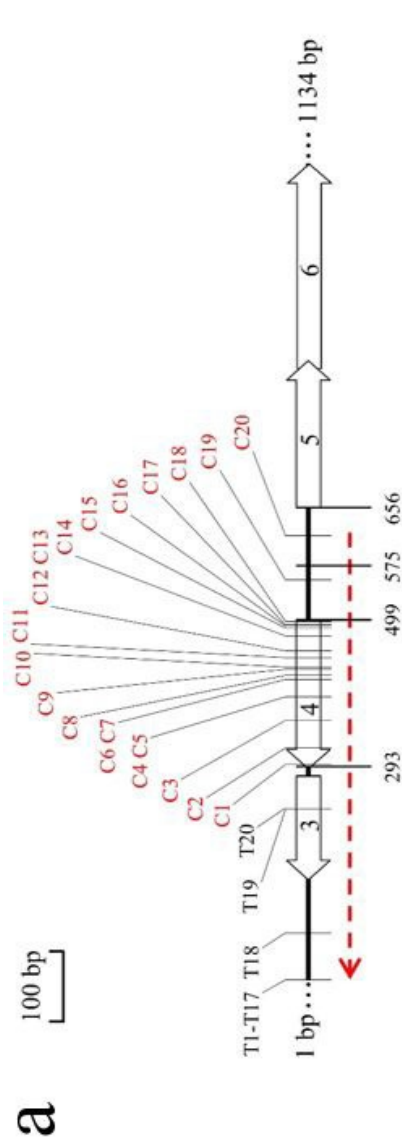
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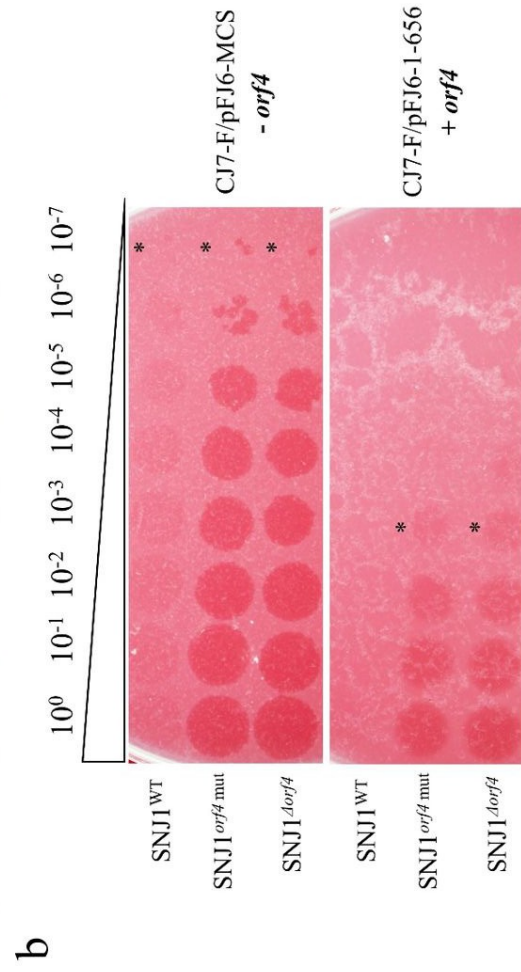
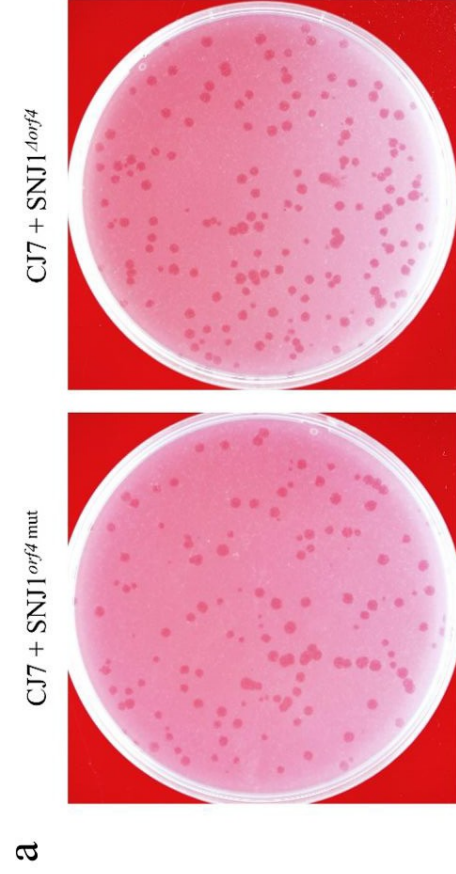
810 **Fig. 7. Alignment of SNJ1 with two SNJ1-like plasmids.** Genomic alignment of

811 SNJ1, *Natrinema versiforme* strain BOL5-4 plasmid pNVE19 (GenBank Acc. No.
812 NZ_CP040333, start from 8,613 bp) and *Haloterrigena jeotgali* strain A29 plasmid
813 unnamed5 (GenBank Acc. No. CP031302, start from 9,455 bp). ORFs of SNJ1 was
814 noted in the arrows. Proteins predicted to be regulators or related to DNA replication
815 were indicated by red or yellow arrows. Capsid proteins defined by mass
816 spectrometric analysis previously(9) were colored orange. Characteristic conserved
817 proteins in family *Sphaerolipoviridae*, including the packaging ATPase, small major
818 capsid protein and large major capsid protein, were marked with pink, light blue and
819 dark blue color (bottom legend). Homologous ORFs in the other two plasmids are
820 shown in the same color, and the percentages of protein identities were shown.

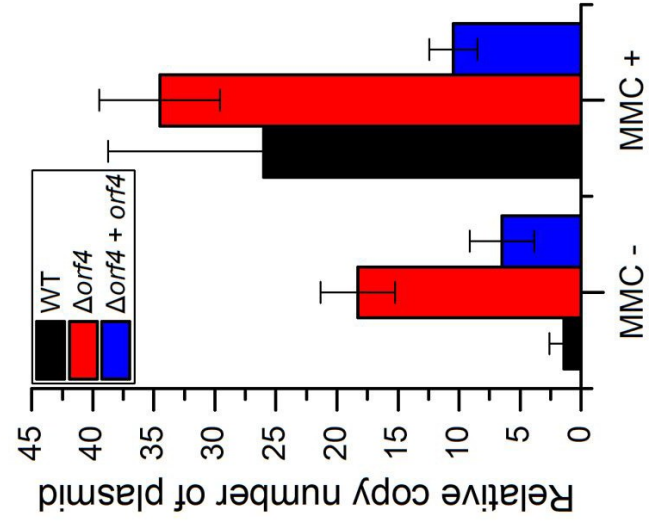
821

822 **Fig. 8. Schematic view of the life cycle of SNJ1.** i : SNJ1 proviral genome resides in
823 J7-1 cytoplasm as plasmid pHH205 with a relative copy number of 1 to 3. ORF4
824 represses expression of the lytic pathway genes presumably by binding to the viral
825 DNA as a dimer, thus maintains SNJ1 in the lysogenic state. ii -v: Upon MMC
826 treatment, ORF4 was inactivated by an unknown mechanism, resulting in expression
827 of the lytic pathway genes and replication of viral genome by the RepA protein. Host
828 cells are lysed and assembled progeny viruses are released into supernatant. vi-x:
829 Released SNJ1 viruses infect CJ7, either enter the lysogenic state as a plasmid or
830 replicate actively, in a process controlled by ORF4. xi : A lysogen of SNJ1 (J7-1) was
831 immune to superinfection of SNJ1 because ORF4 represses replication of the ejected
832 gDNA.





a



b

