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1 **Title: Viruses encode tRNA and anti-retron to evade bacterial immunity**

2

3 Aa Haeruman Azam[1], Kotaro Chihara[1], Kohei Kondo[1], Tomohiro Nakamura[1], Shinjiro Ojima[1],
4 Azumi Tamura[1], Wakana Yamashita[1], Longzhu Cui[2], Yoshimasa Takahashi[1], Koichi Watashi[1],
5 Kotaro Kiga[1,2]

6

7 [1]Research Center for Drug and Vaccine Development, National Institute of Infectious Diseases,
8 Tokyo 162-8640, Japan.
9 [2]Division of Bacteriology, Department of Infection and Immunity, School of Medicine, Jichi
10 Medical University, Shimotsuke-shi, Tochigi 329-0498, Japan.
11 Corresponding author: k-kiga@niid.go.jp

12

13 **Abstract:** Retrons are bacterial genetic retroelements that encode reverse transcriptase capable
14 of producing multicopy single-stranded DNA (msDNA) and function as antiphage defense
15 systems. Phages employ several strategies to counter the host defense systems, but no
16 mechanisms for evading retrons are known. Here, we show that $tRNA^{Tyr}$ and Rad (retron anti-
17 defense) of T5 phage family inhibit the defense activity of retron 78 and a broad range of retrons,
18 respectively. The effector protein of retron 78, ptuAB, specifically degraded $tRNA^{Tyr}$ leading
19 abortive infection, but phage countervailed this defense by supplying $tRNA^{Tyr}$. Rad inhibited
20 retron function by degrading noncoding RNA, the precursor of msDNA. In summary, we
21 demonstrated that viruses encode at least two independent strategies for overcoming bacterial
22 defense systems: anti-defense, such as Rad, and defense canceler, like tRNA.

23 **Keyword:** antiphage defense system, retron, Anti-retron, defense canceler, tRNA.

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The retron defense system composed of reverse transcriptase (RT), non-coding RNA, msrmsd, and accessory protein or RT-fused domain with various enzymatic functions[1–3]. The RT produces satellite msDNA molecules using msd RNA as the template[4]. Following the production of msDNA, the msd RNA template is digested by RNase H[5]. The final product is typically a branched DNA-RNA hybrid in which msd DNA and msr RNA are covalently joined via a 2'-5' phosphodiester bond[4]. In some cases, such as retron Ec78, Ec83 and Sen2, the msd DNA is further separated from the msr RNA[6,7] by the housekeeping exonuclease VII encoding genes *xseA* and *xseB* [6,7]. There are 13 different types of retrons based on their genetic structure and accessory proteins[8]. The accessory protein, which is hugely diverse across different retrons[8], is the executor (effector protein) in retron defense that acts to abort phage infection through the inactivation of bacterial growth. In response to anti-phage defenses, phages have developed various counteract strategies, one of which is to encode proteins that inactivate defense, including these very recently identified anti-BREX[9,10], anti-CBASS[11,12], anti-Pycasr[11], and anti-Thoeris[13]. The arms race between bacteria and phages is the natural driving force of the incessant emergence of sophisticated anti-phage defense systems whose discoveries and mechanistic understandings have brought about multiple impactful modern biotechnological tools.

## Phage genes that inhibit retron function

We have previously isolated and characterized a broad host range *Escherichia coli* phage ΦSP15[14] that shares high similarity with T5j phage, a wildtype T5 from phage collection of Jichi Medical University. ΦSP15 was allowed to undergo spontaneous mutations through passage co-culture with bacteria under Fosfomycin addition. We identified one resultant mutant from each phage - T5n and ΦSP15m, respectively, each carries an approximately 8kb-deletion region in

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47 their genome, which is later found to encode multiple anti-defense systems, and we denoted
48 Anti-Defense Island (ADI) (Fig 1A). The mutant T5n is a T5 strain obtained from Biological
49 Research center, National Institute of Technology and Evolution (Tokyo, Japan) that may have
50 undergone mutation during routine propagation. We evaluated the ability of these four phages to
51 infect a bacterial library encompassing different types of antiphage defense system[3]. Both
52 deletion mutants, T5n and ΦSP15m, showed significant reduction in their infectivity against
53 bacteria carrying Retron Ec67 and Retron Ec78 (Fig 1B and 1C, extended Fig 1). The ADI from
54 ΦSP15 was then divided into nine fragments, each separately cloned into plasmid pKLC23[15]
55 carrying pBAD inducible promoter and transformed into *E. coli* DH10B cells expressing Retron
56 Ec67 or Retron Ec78, revealing that fragment 8 (F8 ADI) could rescue T5n and ΦSP15m from
57 both Retrons whereas fragment 6 (F6 ADI) only rescued phages from Retron Ec78 (Fig 1, D-F,
58 extended Fig 3, A-B, extended Fig 4, A-B). Additionally, we found that F7 ADI and F8 ADI
59 provided protection to phages from another retron that was not used during the first screening,
60 Retron Ec83 (Fig 1F and 1I, extended Fig 5E). We discovered that ORF75 of ΦSP15 which we
61 named rad (retron anti-defense) was the genetic determinant in F8 ADI that enabled the phage to
62 evade the three retrons. Meanwhile, the $tRNA^{Tyr}$ in F6 ADI was responsible for phage rescue
63 from Ec78, and ORF71 and ORF72 of F7 ADI rescued phage from Ec83 (Fig 1, G-I. extended
64 Fig 3c, Extended Fig 4, C-D and F-G, Extended Fig 5).

65

66 **Rad degrades retron ncRNA**

67 Rad is a small protein (189 amino acids) of unknown function, with primase/helicase and
68 TOPIRM/RNase domain (Supplementary Table S1). A search based on homology identified 541
69 Rad homologues in 19,263 phage genomes in the NCBI database. Rad-encoding phages belong

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70 to two families; *Siphoviridae* and *Myoviridae* and infect at least nine different genera of bacteria
71 from three taxonomic phyla; *Proteobacteria, Cyanobacteria*, and *Actinobacteria* (Fig 2A,
72 supplementary Table S2). We demonstrated that the Rad homolog from *Proteus mirabilis* phage
73 ($\text{Rad}^{\text{Proteus phage Privateer}}$) could strongly protect phages from Retron Ec78, while other Rad from
74 *Shigella sonnei* phage ($\text{Rad}^{\text{Shigella sonnei phage}}$) and Salmonella phage vB_Sen_I1 ($\text{Rad}^{\text{Salmonella phage}}$
75 $^{\text{vB_Sen_I1}}$) showed moderate protection (Fig 2B and C, extended Fig 6A). Notably, Rad exhibited
76 extensive inhibition against retrons including retron Ec48 and Se72 (Fig. 2D, Extended Fig 7)[1].
77 The anti-retron activity of Rad showed significant decrease by the introduction of single amino
78 acid mutations at various locations that were conserved in other Rads (R13E, P33T, I88T,
79 D135H, and E156H) (Fig 2, E-F, Extended fig 6B, Extended Fig 8, A-B). And when double
80 mutations at selected conserved amino acids were introduced, Rad defense activity was
81 completely abolished regardless of the location (Fig 2F, extended Fig 8C). Rad was shown to
82 reduce msDNA and ncRNA (msr-msd transcriptional cassettes) of retron, but not the transcript
83 of RT and effector protein, indicating that Rad may degrade the ncRNA to prevent further
84 synthesis of retron (Fig 2, G-H). Exogenous expression of Rad via genome insertion improved
85 T7 infectivity to bacteria carrying retron Ec67 (Fig 2I, Extended Fig 9).
86

87 **ptuAB of retron Ec78 degrades $\text{tRNA}^{\text{Tyr}}$**

88 Since the inhibition of Retron by $\text{tRNA}^{\text{Tyr}}$ was specific to Ec78 (Fig. 1F), we firstly focused on
89 the effector proteins, which show the highest variations in the retron gene cluster. Retron Ec78
90 has two effector proteins, PtuA with an ATPase domain and an HNH endonuclease PtuB[1,3]. We
91 then expressed the effector proteins individually (PtuA or PtuB) or together (PtuAB) under the
92 inducible promoter pBAD. We demonstrated that PtuAB, but not the singly expressed effectors,

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93 triggered bacterial growth arrest, indicating that PtuA and PtuB are the toxins of retron Ec78 (Fig
94 3, A-B). When RT was removed from Retron Ec78, PtuAB toxicity was observed, but not when
95 msrmsd was eliminated, indicating that the antitoxin activity against PtuAB requires RT alone
96 (Fig 3B). This mode of action is different from the tripartite toxin-antitoxin observed in Retron
97 Sen2. RNA hybridization assay showed that the bacterial $tRNA^{Tyr}$ was significantly depleted by
98 PtuAB overexpression (Fig 3, C-D, extended Fig 10A). tRNA sequencing then confirmed that
99 both bacterial $tRNA^{Tyr}$, $tRNA^{TyrU}$ (tRNA-Tyr-GTA-2-2) and $tRNA^{TyrV}$ (tRNA-Tyr-GTA-1-1),
100 were specifically down regulated in the bacteria where PtuAB expression was induced (Fig 3E,
101 extended Fig 10, B-D). Taken together, these results reveal Retron Ec78 exerts its defense
102 mechanism by aborting phage infection through depletion of bacterial $tRNA^{Tyr}$ via PtuAB.

103

## 104 Phage $tRNA^{Tyr}$ cancels abortive infection

105 Because phage derived $tRNA^{Tyr}$ (ΦtRNA-Tyr_SP15) in F6 ADI can rescue phages from Retron
106 Ec78 (Fig 1H), we speculated that ΦtRNA-Tyr_SP15 neutralizes Retron Ec78 through a
107 different mechanism than Rad. Since changing the anticodon sequence of ΦtRNA-Tyr_SP15 or
108 mutating the stem-loop sequence of ΦtRNA-Tyr_SP15 exterminated the neutralization effect of
109 ΦtRNA-Tyr_SP15 (Fig 4, A-E, Extended Fig 11A), we presumed that the function of ΦtRNA-
110 Tyr_SP15 in protein synthesis would be essential for the inhibition of retron defense.
111 Complementation of the exogenous $tRNA^{Tyr}$ by either T5 $tRNA^{Tyr}$ (ΦtRNA-Tyr_T5), *Klebsiella*
112 phage KpP_HS106 $tRNA^{Tyr}$ (ΦtRNA-Tyr_KpP_HS106), and endogenous host bacteria
113 *Escherichia coli* DH10B $tRNA^{Tyr}$ (Ec_tRNA-TyrU or Ec_tRNA-TyrV) *in trans* under the SP15
114 derived tRNA promoter (ΦtRNA-Tyr promoter) successfully restored phage infection to that of
115 ΦtRNA-Tyr_SP15, instead only partial recovery was observed by complementation under *E. coli*

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116 tRNA promoter (Ec_tRNA-Tyr promoter) (Fig 4F, Extended Fig 11B). The ability of $tRNA^{Tyr}$ to
117 rescue phage from retron defense was only observed on retron Ec78 (Fig 4, G-H, Extended Fig
118 11C).
119

120 **Phage sensing mechanism of Retron Ec78 and Retron Ec67**

121 The retron defense system works by sensing phage infection and activating the effector
122 protein(s). Various genetic determinants of phage that triggered antiphage defense systems have
123 been studied elsewhere[16], but only a small number of retron triggers have been identified. We
124 sought to determined how the two retrons, Ec78 and Ec67, recognize and mitigate phage
125 infection through screening for phage mutants that could bypass each of the retrons. T5n,
126 ΦSP15m, and T2 were employed for Retron Ec67, while T5n and ΦSP15m were used against
127 Retron Ec78 (Fig 5A).

128 For Retron Ec78, we found several missense mutations in the gene encoding for exonuclease
129 D15 in all escaper mutants of T5n (seven) and ΦSP15m (four) (Fig 5B). D15 protein catalyzes
130 both the 5'-exonucleolytic and structure-specific endonucleolytic hydrolysis of branched-DNA
131 molecules[22–24]. Co-expression of D15 protein and Ec78 was not toxic to bacteria, but it restored
132 Ec78 defense activity against escaper mutants of T5n and ΦSP15m (Fig 5C, extended Fig 12, A-
133 B). Our findings suggest that Retron Ec78 defense may be triggered by not just D15 protein but
134 also other unknown factor.

135 For Retron Ec67, we found a single point mutation that distinguishes the mutant phages from
136 their parental strains in all seven T5 mutants, four ΦSP15m mutants, and three T2mutants (Fig
137 5B). Since all escaper phages of T2 and T5n/ΦSP15m carry mutations in DenB and protein A1,
138 respectively, we presumed that these genes are the genetic determinants that activate Ec67. Both

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139 protein A1 and DenB are involved in DNA degradation; with protein A1 responsible for the
140 degradation of host DNA as well as the shutoff of host genes[17–19], whereas DenB protein cleaves
141 single-stranded DNA in a dC-specific manner, which may be lethal to host dC-containing DNA
142 replication[20,21]. Co-expression of DenB and Ec67 significantly inhibited bacterial growth (Fig
143 5C). However, we could not observe the effect of protein A1 due to its toxicity[16]. Although T5n
144 and ΦSP15m do not carry DenB homolog protein, DenB complementation restored Ec67 defense
145 activity against not only T2 escaper mutants but also those of T5n and ΦSP15m (Fig 5C,
146 extended Fig 12C). These findings imply that Rectron Ec67 defense may be triggered by the
147 activity of *denB* gene rather than DenB protein itself.
148
149 **Discussion**
150 The current study describes Retron Ec78's defense mechanism and identifies the cellular target of
151 its effector protein PtuAB. These two proteins are also found in Septu[25,26], an antiphage defense
152 system with unknown molecular mechanisms. ATPase-like domain has been found in another
153 nuclease mediated anti-phage defense system Gabija[25,26]. The GbjA protein of Gabija system
154 consists of ATPase-like domain and TOPRIM domain. The ATPase-like domain is strictly
155 regulated by nucleotide concentration *in vitro*, meanwhile the GbjA is activated by the depletion
156 of dNTPs during phage infections, which in turn activates the TOPRIM domain with its nuclease
157 activity, causing bacterial death[27]. We hypothesized that PtuA employs a similar strategy, which
158 may be activated by dNTP depletion during phage infection. This could also explain why D15
159 protein, which is mutated in Ec78 escaper mutant phages, could not activate retron in the absence
160 of phage infection. The expression of either PtuA or PtuB alone is not toxic to bacteria,
161 suggesting these two proteins are most likely working together to induce bacterial growth arrest.

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162 Retron Ec83 also has the same PtuAB effector as Retron Ec78, but failed to protect the phage by
163 tRNA$^{Tyr}$ complementation, suggesting that it is likely targeting other tRNAs or nucleic acids.
164 ADI region is moderately preserved in Tequintavirus (Extended Fig 1B). In SP15, it encodes Rad
165 in the tRNA-rich region, and another retron blocker in F7 ADI that specifically inhibits Retron
166 Ec83 (Fig 1F, extended Fig5). Moreover, the absence of ADI in T5n resulted in lower infectivity
167 against the antiphage defense AVAST 2 (Fig. 1B, extended Fig 1A), indicating the existence of
168 an anti-AVAST 2 in T5n's ADI and supporting the notion that anti-defense genes generally co-
169 localize in a genomic island. Despite the genetic similarity of T-even phages, retron Ec67 used in
170 the current investigation exhibited considerable inhibition on the T2 phage but only moderate
171 inhibition on other T-even phages such as T4 and T6. We hypothesized that T4 and T6 might
172 have other retron blockers, which allow them to evade Ec67. The lack of a Rad homolog protein
173 in those phages, however, suggests that the T-even phage's retron blocker(s), if present, may be
174 different from Rad.
175 First discovered in the 1950s, transfer tRNAs (tRNAs) have been found to play a vital role in the
176 central dogma of molecular biology in all living systems[28,29]. Only one decade after its first
177 discovery, bacteriophages are found to carry their own tRNAs[30]. tRNAs are found in
178 bacteriophage genomes from various bacterial genera[31], but their precise function has long been
179 elusive. Several hypotheses have been proposed, the most well-known is codon compensation, in
180 which codons rarely used by the host but required by the phage are supplemented by phage-
181 encoded tRNAs. This hypothesis is supported by the observation that phage-derived tRNAs tend
182 to correspond with codons that are highly used by phage-encoded genes[32,33]. Recent studies may
183 have hinted at another function of phage-derived tRNAs where they were discovered to be used
184 by phages to counteract the depletion of host tRNAs that occurs as a general response to phage

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185 infection[34,35]. Our data showed that neither phage T5 nor SP15 endured any detrimental effects
186 from losing a significant number of tRNAs in the ADI region. In contrast, these phages are no
187 longer able to infect bacteria that are protected by the Retron Ec78 defense, suggesting that the
188 phage tRNAs are involved in evading bacterial defense. So far, there has been multiple reports
189 about nucleases, such as VapC[36,37], PrrC[38], or RelE[39], of the toxin-antitoxin system, which are
190 also known to target tRNA and are activated by various stress responses, including phage
191 infection[40]. Most bacteria encode multiple defense systems, with an average of ~5 systems per
192 genome[41,42]. This could be one of the reasons why T5-like phages, which carry multiple types of
193 tRNAs in their genome, exhibits exceptionally broad host range[14].
194

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200

201 **Competing interests**

202 A.A.H., Y.T., K.W. and K.K. are co-inventors on a patent pending submitted by National
203 Institute of Infectious Diseases, that based on the results reported in this paper.
204
205
206

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308 **Figure legend**
309
310 **Figure 1.**
311 Identification of phage genes involved in retron evasion. (a) Genomic comparison of T5 and T5-
312 like phage SP15. A genomic region of approximately 8 kb hereafter denotes as Anti-Defense
313 Island (ADI) in T5j and SP15 were missing in T5n and SP15m, respectively. The visualized
314 genomic comparison was generated using Easyfig[1]. (b) Simplified depiction of phage spot assay
315 to evaluate the phage infectivity against bacteria with different antiphage defense systems. Phage
316 solutions from serial 10-fold dilutions were dropped on bacteria lawn and the efficiency of
317 platting (EOP) was measure accordingly. (c) Heatmap depicting the EOP change based on spot
318 assay of phages on bacteria carrying plasmid with different defense systems. The bacterial strain
319 used in this assay was *Escherichia coli* DH10B, the plasmids with antiphage defense systems are
320 provided by Feng Zhang[2] and are available on Addgene. T5n and SP15m showed decreased EOP
321 comparing to their respective wild-type T5j and SP15 on bacteria with retron Ec67 and Ec78
322 defense systems. T5n has decreased EOP comparing to T5j on bacteria carrying AVAST 2. (d)
323 Fragmentation of ADI into nine fragments. The ADI fragments were separately cloned into
324 plasmid under a pBAD inducible promoter and co-transformed with retron into *E coli* DH10B.
325 (e) Genetic organization of fragment 6 (F6 ADI), fragment 7 (F7 ADI), and fragment 8 (F8 ADI).
326 (f) Heatmap based on spot assay of phages on bacteria carrying retron and different ADI
327 fragments. F8 ADI neutralized defense activity of three different retrons Ec67, Ec78, and Ec83,
328 tested in this panel. F6 and F7 ADI specifically neutralized Ec78 and Ec83, respectively. (g, h, i)
329 Heatmap based on spot assay of phages on bacteria carrying retron and different F6, F7, and F8
330 ADI fragments. ORF75 in the F8 ADI neutralized all retrons tested, hereafter we name it Retron-
331 anti defense (Rad). (h) In F6 ADI, $tRNA^{Tyr}$ was found to be the genetic determinant responsible
332 for Ec78 neutralization. The neutralization of F6 ADI was defective when $tRNA^{Tyr}$ was deleted,
333 and co-expression of $tRNA^{Tyr}$ alone with Ec78 neutralized retron Ec78 defense activity. (i)
334 Fragmentation of F7 ADI. Co-expression of two ORF72 and ORF73 are necessary to neutralize
335 retron Ec83. Neither tRNA nor any single gene from F7 could neutralize Ec83. Empty vector
336 pLG001 or pSC101 was used as negative control in all spot assay performed in this figure.
337 Empty vector**s** indicate co-expression of empty vector and plasmid carrying retron.
338
339 **Figure 2.**
340 Rad is a potent blocker of retron defense that is widespread in phage infecting distinct genera. (a)
341 Phylogenetic tree of Rad homologues from diverse bacteria genera. Bacteriophages that infect at
342 least nine genera of bacteria carry Rad homolog, among these the Mycolicibacterium phages
343 made up 40% of the Rad-carrying phages. (b) Simplified depiction of co-expression of Rad and
344 retron. Rad was cloned into plasmid with pBAD inducible promoter. Spot assays were conducted
345 using wildtype phage SP15 or T5j and their corresponding 8 kb deletion mutants, T5n or SP15m.
346 (c) Rad from different phages that infected distinct taxa was capable of neutralizing retron. Rad
347 from Salmonella phage vB_Sen_I1 and *Shigella sonnei* phage only slightly impair retron defense,
348 whereas Rad from SP15, T5, and proteus phage Privateer all demonstrated substantial inhibitory
349 action. (d) Rad blocks different retrons of distinct group. Rad effectively blocked retron Ec67,
350 Ec78, and Ec83 and rescued SP15m. Utilizing phage λ vir, Rad rescued the phage from retron
351 Ec48 and Se72. (e) Predicted structure of Rad using Alphafold. Amino acid mutations were
352 introduced into conserved residues in Rad at different locations. (f) Single amino acid mutation
353 in all selected locations slightly reduced activity of Rad to block retron. Rad activity was

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354 completely hampered when each of the selected amino acid mutant was introduced together. (g)
355 TBE-Urea PAGE of extracted msDNA from bacteria expressing retron. The msDNA product
356 was significantly reduced when Rad was co-expressed with retron. Same result was observed in
357 three different retrons Ec67, Ec78, and Ec83. (h) Real time quantitative PCR of retron cassette
358 (msr-msd, RT, and effector protein) of Ec78. The relative expression of msr and msd RNA were
359 significantly lower when Rad is co-expressed with retron. The experiment was conducted on
360 three independent samples. Asterix indicates significant difference (****P*<0.01, **P*<0.05,
361 according to student t-test). (i) Exogenous expression of Rad in T7 phage. Chimera T7 carrying
362 *rad* gene (T7rad) exhibited increased infectivity by tenfold against bacteria carrying retron Ec67.
363
364 **Figure 3.**
365 $tRNA^{Tyr}$ is the cellular target of retron Ec78's effector proteins. (a) Simplified depiction of
366 method to evaluate cellular target of PtuAB of Ec78. The effector protein of Ec78 (PtuAB) was
367 cloned individually, PtuA or PtuB, or together, PtuAB, into plasmid under pBAD promoter.
368 Glucose was used to block pBAD promoter while arabinose was used to induce the promoter.
369 The induced cell was evaluated for their cytotoxicity (b), and the reduction in the expression of
370 $tRNA^{Tyr}$ by dot blot RNA hybridization (c and d) and tRNA sequencing (e). (b) Induction of
371 PtuAB promotes bacterial growth arrest. Induction of PtuA or PtuB alone was not toxic to
372 bacteria, whereas induction of both (PtuAB) was toxic. (c and d) $tRNA^{Tyr}$ were significantly low
373 in the bacteria where PtuAB was expressed. The intensity of the dot obtained from the RNA
374 hybridization assay was visualized using ImageJ (d). (e) tRNA sequencing revealed the $tRNA^{Tyr}$
375 was significantly down regulated when PtuAB was induced.
376
377 **Figure 4.**
378 $tRNA^{Tyr}$ from other phages or from host bacteria could rescue phage from retron Ec78. (a)
379 Simplified depiction of the method to evaluate tRNA complementation on bacteria carrying
380 retron Ec78. Complementation of tRNA was performed *in trans* by cloning the tRNA into
381 plasmid under phage SP15 derived tRNA promoter (ΦtRNA-Tyr promoter) located in F6 ADI.
382 (b) RNAFold[3]-based structural prediction of $tRNA^{Tyr}$ SP15 (ΦtRNA-Tyr_SP15). To assess the
383 impact of the mutation on the restoration of the phage from retron Ec78, sequence and structural
384 mutations were introduced into tRNA. These mutations included the CCA terminus (CCA into
385 AAA, yellow-green box), acceptor stem (UGG into AAA, orange box), D-stem (UGG into AAA,
386 violet box), anti-stem (GUC into AAA, blue box), and T-stem (GGU into AAA, green box). (c)
387 Phylogenetic tree of $tRNA^{Tyr}$ used in this study. (d) Sequence alignment of $tRNA^{Tyr}$ from T5
388 (ΦtRNA-Tyr_T5), SP15 (ΦtRNA-Tyr_SP15), *Klebsiella* phage KpP_HS106 (ΦtRNA-
389 Tyr_KpP_HS106), and *E. coli* tRNATyr (Ec-tRNA_TyrU or Ec-tRNA_TyrV). According to the
390 predicted secondary structure, the loop, stem, and anticodon sequence of SP15 were highlighted
391 in red letters within the colored boxes. (e) Heatmap based on spot assay of phage SP15 and
392 SP15m on bacteria carrying retron Ec78 complemented with different mutant of ΦtRNA-
393 Tyr_SP15. Regardless of the mutation locations, $tRNA^{Tyr}$ mutations eliminated the $tRNA^{Tyr}$'s
394 capacity to neutralize retron defense. (f) $tRNA^{Tyr}$ from different phages (ΦtRNA-Tyr_T5 and
395 ΦtRNA-Tyr_KpP_HS106) or from *E coli* rescue phage from retron Ec78. Notably,
396 Ec_tRNA_Tyr could rescue the phage to the same extent as ΦtRNA-Tyr_SP15 when SP15
397 tRNA promoter was utilized, however when *E. coli* tRNA promoter was used (Ec_tRNA-Tyr
398 promoter), the retron defensive activity was still visible. Other tRNAs (ΦtRNA-Phe_SP15 or
399 Ec_tRNA-His_DH10B) and tRNATyr from human (Hs_tRNA_Tyr) or *Staphylococcus aureus*

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400 (Sa_tRNA_Tyr_USA300) did not rescue phage from retron Ec78. (g and h) $tRNA^{Tyr}$ specifically
401 rescued phages from retron Ec78. Co-expression of $tRNA^{Tyr}$ with other retron was not able to
402 rescue phage SP15m from retron Ec67, Ec78, nor Ec83 (g). Similarly, $tRNA^{Tyr}$ was not able to
403 rescue phage $\lambda$ vir from Ec48 nor Se72 (h).
404
405 Figure 5.
406 Evaluation of retron trigger. (a) Screening of retron escaping phages adapted from another study[4]
407 with some modifications. Phage and bacteria harboring retron were cocultured in liquid medium
408 overnight. Escaping mutant phages were screened from phage mixture obtained from the co-
409 culture. Such mutant phages should form single plaque even when retron is presented in bacteria.
410 The genomes of selected escaping mutans were analyzed and mapped to parental phage genome.
411 Shared mutations in all escaping phages are expected to be the phage component that
412 desensitized the phage to retron defense. (b) Mutations identified in escaping mutant phages. (c)
413 Phage genes that are commonly mutated in escaping phages were tested for their toxicity when
414 co-expressed with retron. D15 protein that was mutated in all Ec78 escaping phages was not
415 toxic even when co-expressed with retron Ec78. DenB protein from Ec67 escaping T2 phage was
416 highly toxic when co-expressed with Ec67, suggesting the importance of the gene in the
417 activation of Ec67. (d) Complementation of D15 restored retron defense against escaping phages,
418 T5-8e andSP15m-8e, whereas complementation of the mutated version of D15 did not restore
419 retron activity, indicating D15 may involve in retron Ec78 activation. (e) DenB complementation
420 restored Ec67 defense against the escaping phages T5-6e, SP15m-6e, and T2-6e. Co-expression
421 of DenB without ATc induction could restore retron defense against escaping phage T5-62 and
422 SP15m-6e, but induction with 50ng/ml ATc (DenB 50) was needed to see the change in the
423 escaping phage T2-6e. However, in such condition, retron Ec67 defense activity was not visible
424 against escaping phage T5-6e and SP15m-6e, perhaps due the high toxicity of DenB. (f)
425 Proposed mechanism of phage to escape retron Ec78. Phages encode three genetic factors related
426 to retron defense; Retron-anti defense (Rad) that inhibited retron biosynthesis, retron trigger
427 (D15 for Ec78, DenB or A1 for Ec67), and tRNAs to supplement the host tRNAs that was
428 degraded by retron Ec78. Following infection of phage, retron may sense the phage protein that
429 is either pre-made and packed together with phage genome in the capsid or the protein that is
430 expressed during early production of phage particle. Production of such sensor/trigger may
431 activate retron defense system either due to degradation of retron component (because most
432 retron triggers identified in the current study are interestingly involved in nucleotide degradation)
433 or retron senses degradation of host genomic DNA. Once the retron is activated, effector protein
434 will be released from retron complex. We suggested that the depletion of dNTPs due to the
435 overused of those for phage assembly may further trigger conformational change of PtuAB to its
436 active form that cleaves bacterial tRNAs, resulting in bacterial growth arrest and aborts phage
437 production. To evade retron defense, however, phage is equipped with Rad and/or, in case of
438 Ec78, the phage encoded tRNAs as the counter-agents of PtuAB effector protein.
439

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Fig.1

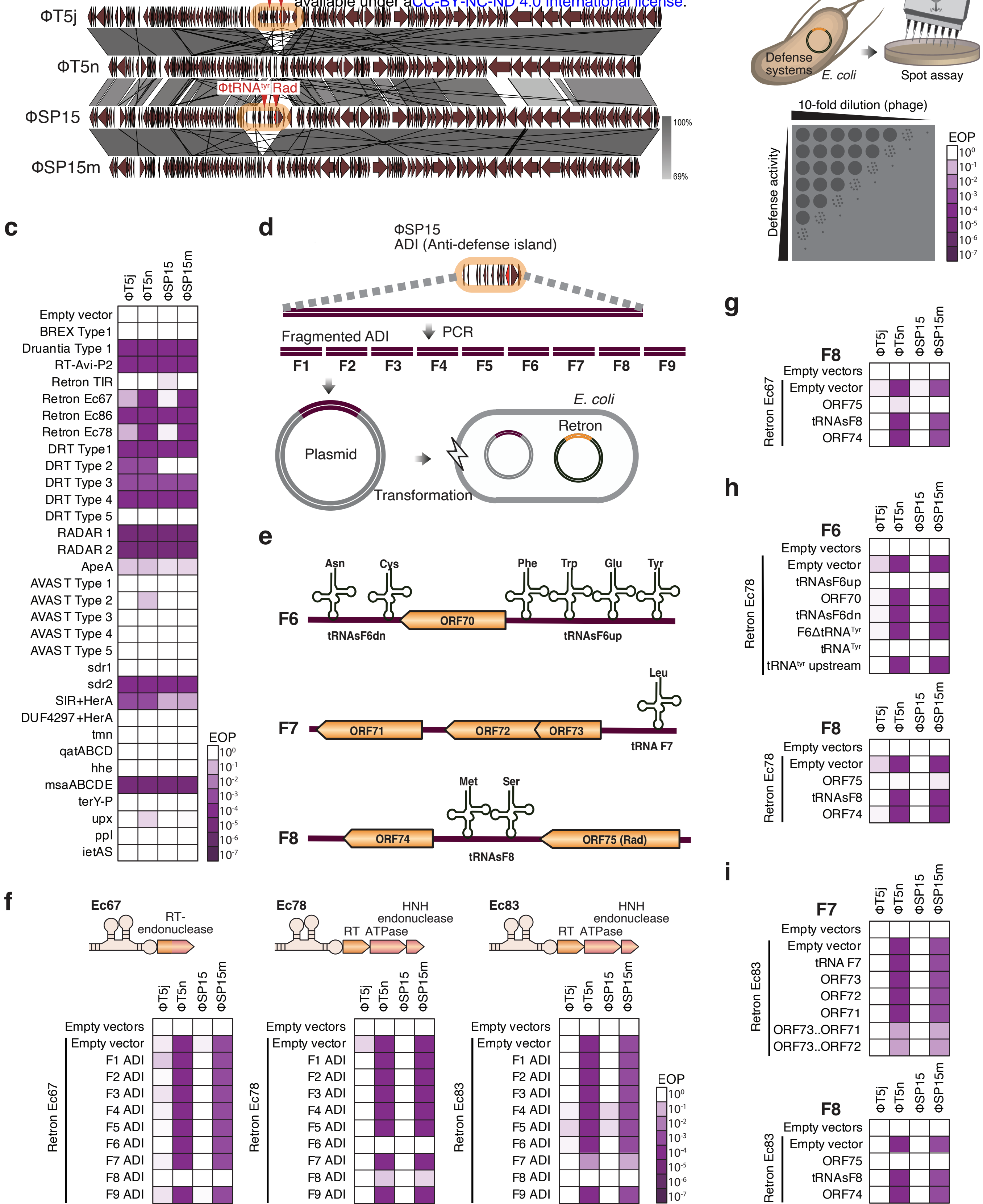

Fig.2

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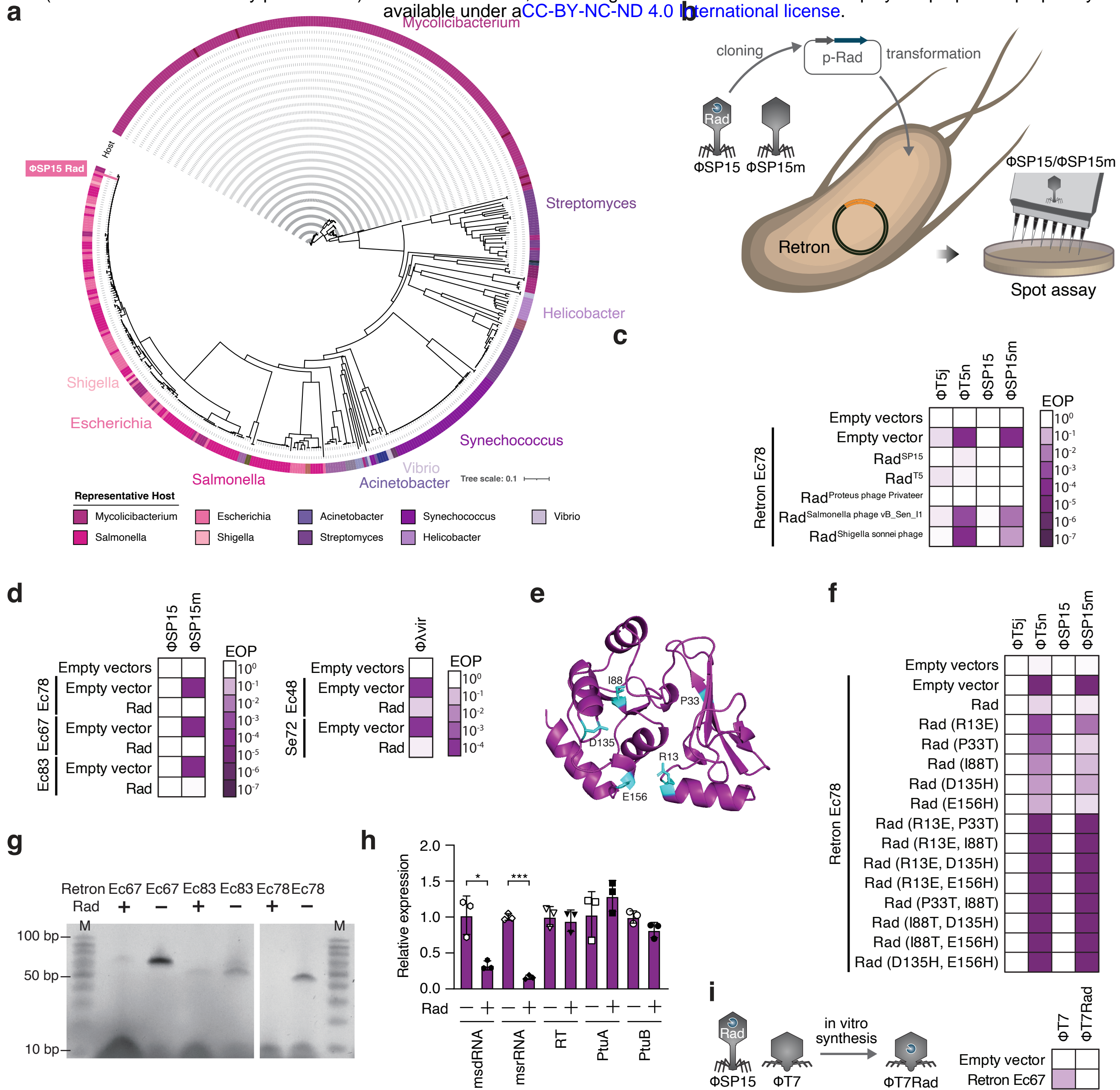

Fig.3

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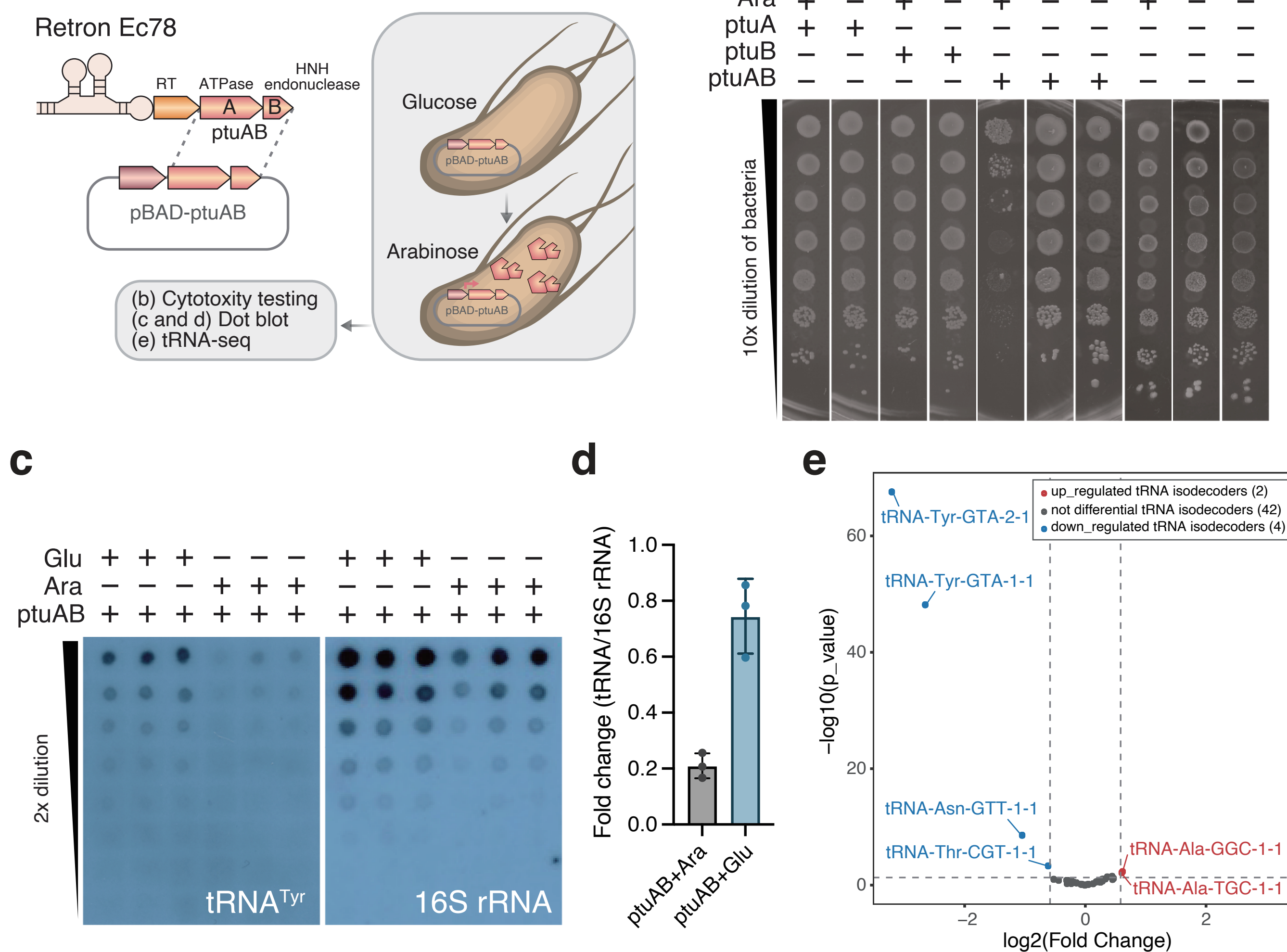

Fig.4

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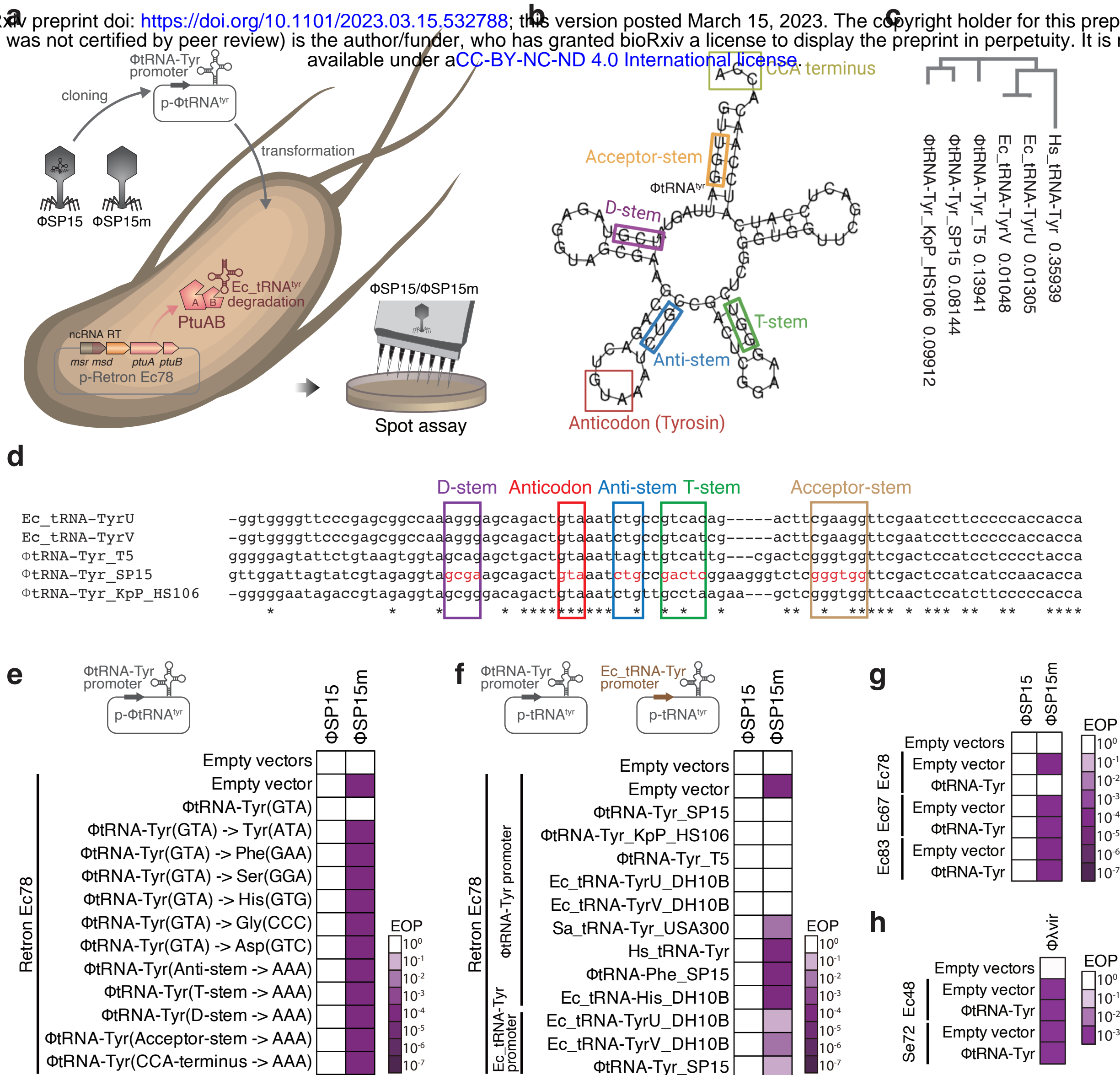

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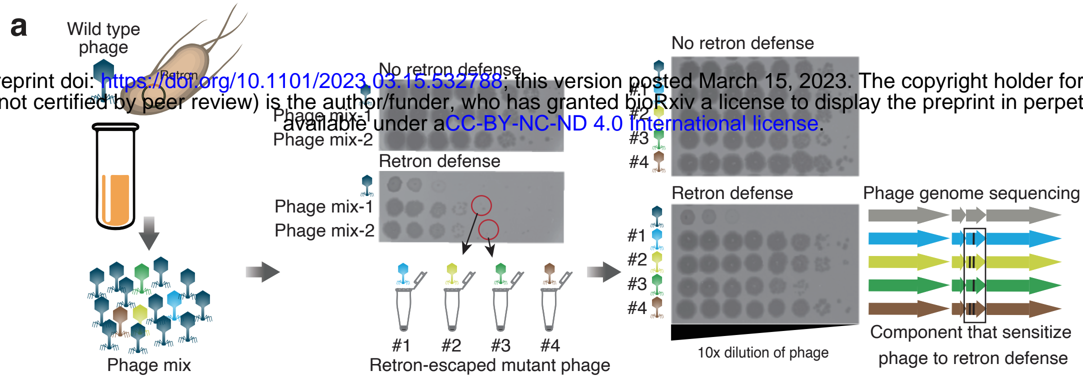
# Fig.5

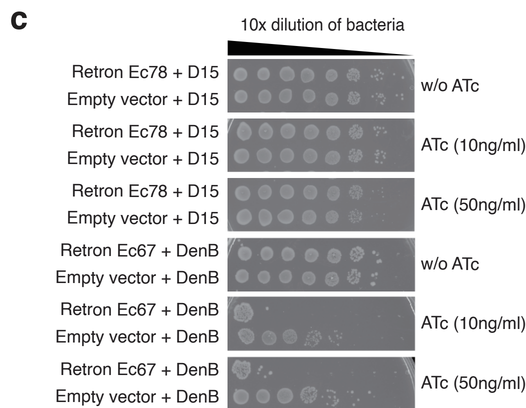
a



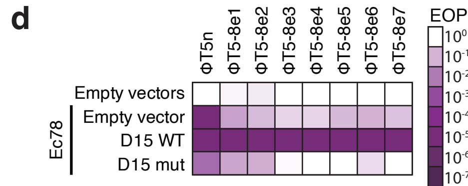

b

| Mutant phage | Sensitivity to Retron Ec78 | Sensitivity to Retron Ec67 | Mutated gene: Flap endonuclease D15 | DNA-binding D2 | HNH endonuclease HegF | Endonuclease DenB | ssDNA binding | Protein A1 | Long tail fiber |
|---|---|---|---|---|---|---|---|---|---|
| ΦT5_8e1 | Resistance | | Lys83Asn | | Ala101Asp | | | | |
| ΦT5_e82 | Resistance | | Arg86His | Ala78Pro | | | | | |
| ΦSP15m_e81 | Resistance | | Arg86Leu | | | | | | |
| ΦSP15m_e82 | Resistance | | Arg86Gln, Gly154Ser | | | | | | |
| ΦT5_6e1 | | Resistance | | | | | | Frameshift | Gly1057Ala |
| ΦT5_6e2 | | Resistance | | | | | | Frameshift | |
| ΦSP15m_6e1 | | Resistance | | | | | | Frameshift | |
| ΦSP15m_6e2 | | Resistance | | | | | | Gly516Asp | |
| ΦT2_6e1 | | Resistance | | | | Frameshift | Thr166Asn | | |
| ΦT2_6e2 | | Resistance | | | | Frameshift | | | |

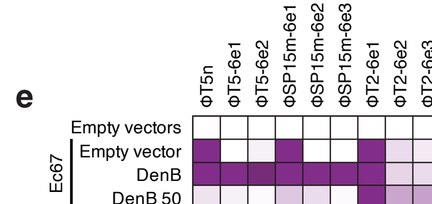
c




d




e




f