

Survey of lysogenic phages in the 72 strains of *Escherichia coli* collection of reference (ECOR) and identification of a phage derived from the ECOR52 strain

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To cite this article:

Yuka Shibata, Chisato Ugumori, Anna Takahashi, Ayuka Sekoguchi, Sumio Maeda. Survey of Lysogenic Phages in the 72 Strains of *Escherichia coli* Collection of Reference (ECOR) and Identification of a Phage Derived from the ECOR52 Strain. *American Journal of BioScience*. Vol. 2, No. 2, 2014, pp. 32-37. doi: 10.11648/j.ajbio.20140202.12

Abstract: *Escherichia coli* collection of reference (ECOR) is a standard collection of 72 wild-type *E. coli* strains that represent natural *E. coli* populations found in various environments. Although these strains are widely used in experiments investigating the physiology and behavior of wild-type *E. coli*, their genetic features including accessory DNA have not been sufficiently studied. In this study, we surveyed for the presence of lysogenic phages in each ECOR strain under both inducing and non-inducing conditions. We found that 34 strains could produce plaque-forming phages; among them, 14 strains were newly discovered to harbor lysogenic phages capable of entering the lytic cycle. We isolated a new phage (designated as "MSU52-L1") from the ECOR52 strain and identified it as a P22/lambda-like phage with homology to known phages, such as CUS-3, HK620, and HK140.

Keywords: Bacteriophage, *Escherichia coli* Collection of Reference, Lambdoid Phage, P22

1. Introduction

Escherichia coli, one of the most extensively studied bacteria, is used worldwide for scientific and biotechnological experiments. Most of the *E. coli* strains generally used are "laboratory strains" derived from the *E. coli* K-12 strain [1]. The *E. coli* collection of reference (ECOR), established from 2,600 natural isolates by Ochman and Selander [2], is a set of 72 standard strains representative of the natural *E. coli* populations in the environment. The ECOR strains have been widely used in various studies on the physiology, behavior, and genotypic variation of wild-type *E. coli* [3]. While some genetic features of the individual ECOR strains have been analyzed [3], their accessory DNA have not been sufficiently studied [4, 5, 6], with only a few reports on lysogenic phages in the ECOR strains. Riley and Gordon [7] showed that 24 of the colicin-producing ECOR strains may possess unknown inducible phages. Nilsson *et al.* [8] demonstrated by hybridization and PCR experiments that 26 ECOR strains carry portions of the genomic DNA sequence of a P2-like phage, although production of active phages was not

demonstrated. Sandt *et al.* [9] found that the ECOR9 strain harbors prophages containing genes coding for immunoglobulin-binding proteins.

In this study, we surveyed for the presence of lysogenic phages in all the 72 ECOR strains under both inducing and non-inducing conditions. We present the results of the screen for lysogenic phages and a sequence analysis of the phage (designated as MSU52-L1) isolated from the ECOR52 strain.

2. Materials and Methods

2.1. *E. coli* Strains, Plasmids and Materials

The 72 ECOR strains were kindly provided by Prof. Thomas S. Wittam (Michigan State University, USA). MG1655 (F⁻, λ ⁻, *ilvG*⁻, *rfb-50*, *rph-1*; [10]), pHSG299 [11], and pUC19 [12] were obtained from the National BioResource Project (NIG, Japan): *E. coli* (<http://www.shigen.nig.ac.jp/ecoli/strain/top/top.jsp>).

Luria–Bertani medium (LB; Lennox) was purchased from Sigma (St. Louis, USA). Distilled water (DNase- and RNase-free, molecular biology grade) and kanamycin were obtained from Invitrogen (Carlsbad, USA). DNase I (bovine pancreas, Grade II) was obtained from Roche Diagnostics (Mannheim, Germany). KAPA Taq Extra (KAPA Biosystems) was purchased from Nippon Genetics (Tokyo, Japan). Syringe filters for removal of cells (pore size: 0.20 µm) were obtained from Iwaki (Tokyo, Japan). Amicon Ultra-4 was purchased from Millipore (Darmstadt, Germany). Agar (guaranteed reagent grade), ampicillin, and other general reagents were obtained from Wako (Tokyo, Japan).

2.2. Spot Assay for Phage Detection

Spot assay was performed by a conventional method [7, 13]. In brief, after overnight pre-culture, each ECOR strain was cultured in LB broth in the presence or absence of mitomycin C (0.2, 1, or 3 µg/ml) at 37°C for 5 h in a shaker. The culture supernatant was obtained by centrifugation (5,000g, 10 min) and filtered using a membrane filter (pore size: 0.20 µm) to remove residual cells. An aliquot (10 µl) of this supernatant was spotted on LB top agar containing MG1655 as the phage-infection host strain in a polystyrene dish (90 mm). After overnight incubation at 37°C, clear lysis zones were observed.

2.3. Plaque Assay for Phage Detection

Plaque assay was performed according to a previously described method [14]. In brief, the culture supernatant of

each ECOR strain was prepared as described earlier. To obtain optimal plaque formation under inducing conditions, mitomycin C was used at 0.2 µg/ml for most strains and at 3 µg/ml for the ECOR26 strain. An aliquot (10 µl) of the culture supernatant was mixed with LB soft agar containing MG1655 as the phage-infection host strain and spread onto LB agar dishes (90 mm). After overnight incubation at 37°C, plaques were observed and counted.

2.4. Isolation of a Phage from the ECOR52 Strain and Purification of its DNA

Phage particles derived from the ECOR52 strain were amplified and isolated according to a previously described method [14]. For efficient harvest of phage particles, the ECOR52 strain was cultured under inducing conditions. In brief, the ECOR52 was incubated at 37°C for 5 h in 100 ml of LB broth in the presence of mitomycin C (0.2 µg/ml). The culture was centrifuged (5,000g, 10 min), and the resulting supernatant was filtered as described earlier. The supernatant was treated with DNase I (5 µg/ml) and RNase A (60 µg/ml) to remove the *E. coli* nucleic acid contaminants. Phage particles in the supernatant were concentrated by ultrafiltration using Amicon Ultra-4 (30 K; 7,500g, 30 min) and washed with SM buffer [50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 8 mM MgSO₄, 0.01% gelatin]. Phage particles on the membrane were recovered with 1 ml of SM buffer, and phage DNA was purified by phenol/chloroform (1:1) extraction and ethanol precipitation.

Table 1. PCR Primers used for MSU52-L1 phage identification.

Phage	Target Gene (target sequence)	Primer Name	Primer Sequence (5'-3')	Size of PCR Product (bp)
CUS-3	protein O (30973–31159)	CUS-3 O-L	ACCGACTTCGGTGTGTAGG	187
		CUS-3 O-R	ACATGAAAGCGCACATGAAG	
	<i>neuO</i> (1555–1774)	CUS-3 neuO-L	AATATTGGTAATATGTCTGCATGATG	220
		CUS-3 neuO-R	GTTGGAAGCGGATCTGTCAT	
HK620	protein 5 (capsid protein) (25401–25554)	HK620 5-L	TGTATAACGGAGCCACACCA	154
		HK620 5-R	GCCGCTACCTGACGACTTAC	
mEp043 c-1	DNA replication protein O (33729–33966)	mEp043 O-L	TTGCAAATGCCCTGTATGAA	238
		mEp043 O-R	TTCTTGTTAGGCCCGATTG	
	<i>cI</i> (32446–32612)	mEp043 C1-L	ACCCGCACGAACGTTACC	167
		mEp043 C1-R	GAATGCTGTATCCGGAAGGA	
HK97	protein O (30043–30195)	HK97 O-L	ACGGATTCAATAAGCCGATG	153
		HK97 O-R	CCACCTTGTTCACTCCGATT	
	<i>cro</i> (29053–29253)	HK97 cro-L	ATGGAACAACGCATAACCCCTG	201
		HK97 cro-R	TTATGCAGTTGTTTTTTGTT	
HK022	<i>cI</i> (28610–28763)	HK022 C1-L	TGCCATCGCCATCAAAAC	154
		HK022 C1-R	TATACGACCGAGCAGGCAAG	
HK633	truncated prophage repressor (30250–30405)	HK633 TPR-L	ACTTACGCCAAGAGCTGACG	156
		HK633 TPR-R	TCCAGGGTAAAAAGCAAAAGAA	
HK140	DNA replication protein O	HK140 O-L	CATCAGCCAGAAAACCGAAT	207

Phage	Target Gene (target sequence)	Primer Name	Primer Sequence (5'-3')	Size of PCR Product (bp)
mEpX1	(32831–33037)	HK140 O-R	CGACTGCCTGTTGCTTGTTA	161
	DNA replication protein P (33201–33361)	HK140 P-L	CAGGTAGCGCAGATCATCAA	
		HK140 P-R	CCTGCGTTAACCTGTTCCAT	
	putative replication protein DnaC (33284–33485)	mEpX1 DnaC-L	CCTGGAACCTGGAAGAACCA	202
		mEpX1 DnaC-R	CGCCAACCTCATCGATTATT	
	<i>cI</i> (30802–31000)	mEpX1 C1-L	CCTTCACCTCAAGCCAGAAA	199
mEpX2	exonuclease (22881–23090)	mEpX1 C1-R	GCACTCGGCGTATCTCCG	
		mEpX2 exo-L	GTTTGTGGTTGGCGTTCTTT	210
		mEpX2 exo-R	CGAATACCGGAGAAACAGGA	

2.5. Cloning, Sequencing, BLAST, and PCR Analyses of the ECOR52-phage Genomic DNA

Purified ECOR52-phage genomic DNA was digested with EcoRI or HindIII, and the resulting 9 fragments were cloned into pHSG299 or pUC19. Both strands of these 9 fragments were sequenced, resulting in 18 sequences of approximately 400–800 bp each (Accession number: AB841350–AB841367). These sequences were analyzed using BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

PCR primers (Table 1) were designed on the basis of the genome sequences of 9 P22-like or lambdoid phages [CUS-3 (Accession number CP00071); HK620 (Accession number AF335538); mEp043 (Accession number JQ182734); HK97 (Accession number AF069529); HK022 (Accession number AF069308); HK633 (Accession number JQ086377); HK140 (Accession number JQ086370); mEpX1 (Accession number JQ182727); and mEpX2 (Accession number JQ182726)]. KAPA Taq Extra enzyme and MSU52-phage genomic DNA template were used for PCR, which included an initial denaturation step of 3 min at 95°C, 35 cycles of 30 s at 95°C, 30 s at 60°C (−0.1°C per cycle), 40 s at 72°C, and final extension of 5 min at 72°C. PCR products were analyzed by conventional 0.8% agarose/tris–borate–EDTA (TBE) gel electrophoresis.

3. Results and Discussion

3.1. Screening for Lysogenic Phages from Culture Supernatants of the Individual ECOR Strains under Non-inducing Conditions and Inducing Conditions with Mitomycin C

At first, screening for lysogenic phages from culture supernatants of the 72 individual ECOR strains was performed under non-inducing conditions. The produced phages were tentatively detected with a spot assay using MG1655 as the host strain and confirmed with a subsequent plaque assay. Plaques were detected from 24 ECOR strains; among them, 9 strains (strain numbers: 1, 16, 19, 20, 49, 52, 68, 69, and 70) were newly identified to produce active phages. The number, clarity, and diameter of the plaques are summarized in Table 2.

Next, screening for lysogenic phages was performed

under inducing conditions using mitomycin C to identify the lysogens that were not detected under non-inducing conditions. Plaques were detected from 10 ECOR strains

Table 2. Results of plaque assay under non-inducing and inducing conditions.

ECOR Strain No.	Number	Plaque Clarity	Diameter
Non-inducing Conditions			
1	+	C	S
3	+	C	S
4	+	C	S
5	+	C	S
7	+	C	S
10	+	C	S
11	+	C	M
12	++	C	S
13	+	C	S
15	+	C	S
16	+	C	S
19	+	C	S
20	+	C	S
25	+	C	S
43	++	C	S
44	+	C	M
48	+	C	S
49	+	C	M
52	+	C	S
53	++	T	S, M, or L
59	+++	T	S, M, or L
68	+	C	S
69	++	T	L
70	++	T	M
Inducing Conditions			
24	+	C	M
26	+++	T	S or M
27	+	C	S
36	+	C	M
40	+	T	M
41	++	C	S
47	+	T	S
51	+	C	M
71	+	T	M
72	+	T	M

Number: +, <10; ++, 10–99; +++, ≥100. Clarity: C, clear; T, turbid. Diameter (mm): S, <0.5; M, 0.5–1; L, >1.

(Table 2); among them, 5 strains (strain numbers: 26, 27, 47, 51, and 71) were newly identified to produce active phages. The number, clarity, and diameter of these plaques

are also summarized in Table 2.

Taken together, we found 34 phage-producing strains, and 14 of them were newly discovered to harbor lysogenic phages. Although Sandt *et al.* [9] previously described that the ECOR9 strain harbors prophages, we could not detect plaque-forming phages from the culture supernatant of the ECOR9 strain under the conditions we adopted. Therefore, further detailed experiments involving various conditions of

culture, phage induction, and host strains in plaque assay will be required for a thorough screening for lysogenic phages, which are capable of producing active phage, in the ECOR strains.

3.2. Grouping of Phage-producing ECOR Strains on the Basis of their Plaque Characteristics and Phylogenetic Groups

Table 3. Grouping of phage-producing ECOR strains on the basis of their plaque characteristics and phylogenetic groups.

Plaque Number	Plaque Clarity	Plaque Diameter (mm)		
		<0.5	0.5–1	>1
1–9	Clear	[A] 1, 3, 4, 5, 7, 10, 13, 15, 16, 19, 20, 25 [B1] <u>27</u> , 68 [B2] 52 [D] 48	[A] 11, <u>24</u> [B2] <u>51</u> [D] <u>36</u> , 44, 49	
	Turbid	[D] <u>47</u>	[B2] <u>71</u> , <u>72</u> [D] <u>40</u>	
10–99	Clear	[A] 12 [D] <u>41</u> [E] 43		
	Turbid	[B2] (53)	[B1] 70 [B2] (53)	[B1] 69 [B2] (53)
≥100	Clear			
	Turbid	[B1] (<u>26</u>) [B2] (59)	[B1] (<u>26</u>) [B2] (59)	[B2] (59)

Phage-producing ECOR strains are represented by their strain numbers, subsequent to respective phylogenetic-group names: [A], [B1], [B2], [D], and [E]. Strains that produced plaques under inducing conditions are underlined. Strains that produced plaques of multiple sizes are shown in parentheses.

Table 3 shows grouping of phage-producing ECOR strains on the basis of their plaque characteristics (number, clarity, and diameter of the plaques that they produced) and phylogenetic groups. The ECOR strains were distributed into 11 plaque-characteristic groups. Among them, almost half of the strains (16 of 34) belonged to the plaque group of “small-number (1–9), clear, and small-diameter (<0.5 mm),” suggesting that many ECOR strains commonly possess a similar type of phage. In other strains, such as the ECOR26, 53, and 59 produced plaques of multiple sizes, suggesting that they harbor multiple types of lysogenic phages or contain sub-populations of cells showing heterogeneity in phage production.

The ECOR strains are known to be classified into 5 phylogenetic groups [group A (25 strains); group B1 (17 strains); group B2 (14 strains); group D (12 strains); group E (4 strains)] (<http://www.shigatox.net/new/reference-strains/ecor.html>). A comparison of this phylogenetic grouping with the grouping based on plaque characteristics (Table 3) revealed that most of the strains (12 of 16) in the plaque group of “small-number, clear, and small-diameter” belonged to the phylogenetic group A, and that all strains in the plaque group of “number >10 and turbid” belonged to the phylogenetic groups B1 or B2. These results suggest that respective phylogenetic groups of the *E. coli* tend to harbor particular types of phages. Other features of each ECOR strain, such as the source of isolation, showed no apparent correlation with the presence/absence of phage and plaque type.

A previous study by Nilsson *et al.* [8] showed that 26 ECOR strains carry portions of the genomic DNA sequence

of a P2-like phage, although production of active phages was not demonstrated. In our study, 12 strains (strain numbers: 4, 5, 7, 10, 12, 13, 15, 43, 44, 48, 53, and 59) that were identified are included in Table 3, but 13 strains were not identified. Among the 12 identified strains, 7 (strain numbers: 4, 5, 7, 10, 13, 15, and 48) belonged to the group of “small-number, clear, and small-diameter,” whereas the other 5 strains belonged to other plaque-type groups. Therefore, it appears that possession of the genomic DNA sequence of a P2-like phage does not directly correlate with active phage production and plaque type, although some correlation with plaque type may exist.

3.3. Isolation and Characterization of a Phage Derived from the ECOR52 Strain

We next attempted to isolate and identify some of the phages and succeeded in identifying a phage derived from the ECOR52 strain (plaque type: “small-number, clear, and small-diameter” and phylogenetic group: B2). The phage particles were purified from the culture supernatant of the ECOR52 strain, and its genomic DNA was isolated and digested with EcoRI or HindIII. The resulting 9 fragments were cloned into cloning vectors and partially sequenced. The obtained DNA sequences (Accession number: AB841350–AB841367) of total approximately 9-kb length were analyzed using BLAST search; the results are summarized in Table 4. The cloned sequences were highly homologous to 15 known phages, most of which belonged to the P22 and lambdoid phage groups.

Table 4. Summary of the results of the BLAST search using genomic DNA fragments of phage MSU52-L1.

Phage Family	Phage Name	Fragment	1500		4000		7000		1000		1100		
		Direction	L	R	L	R	L	R	L	R	L	R	
		Query bp	470	476	901	871	486	525	486	488	803	488	
P22	CUS-3	QC	100	100			37		76	46	31	24	
		MI	91	85			96		97	98	99	98	
	HK620	QC							76	98	31	24	
		MI							97	98	98	94	
	g341 c	QC									53	84	
		MI									98	77	
	P22	QC					99				53		
		MI					90				97		
	Sf6	QC											
		MI											
	Lambda	mEp043 c-1	QC							99	98		
			MI								98	98	
HK140 & others*		QC							76	98			
		MI								95-	98-		
Other phages	RE-2010	QC						76					
		MI						85					
	Bups phi1 clone3	QC											
		MI											
Others	<i>E. coli</i> O104: H4	QC										59	
		MI										98	
	2009EL-2071	QC											
		MI											
	<i>E. coli</i> P12b	QC											
		MI											

Table 4. Continued.

Phage Family	Phage Name	Fragment	1900		2400		3000		8000		
		Direction	L	R	L	R	L	R	L	R	
		Query bp	485	487	500	471	486	485	482	486	
P22	CUS-3	QC						99	25	98	
		MI						98	97	94	
	HK620	QC					100	68	83	98	
		MI					96	91	93	93	
	g341 c	QC					98	68	26		
		MI					96	98	92		
	P22	QC		98			98		23		
		MI		99			94		92		
	Sf6	QC	56						83	98	
		MI	77						95	95	
	Lambda	mEp043 c-1	QC					100			
			MI					98			
		HK140 & others*	QC					99-			
			MI					97-			
	Other phages	RE-2010	QC					98			
			MI								
Bups phi1 clone3		QC			31						
		MI			72						
Others	<i>E. coli</i> O104: H4	QC									
		MI									
	2009EL-2071	QC									
		MI									
	<i>E. coli</i> P12b	QC	79								
		MI	97								

Accession numbers of the DNA sequences of the fragments used for BLAST search: AB841350–AB841367. QC: query coverage (%); MI: max identity (%). others*: HK022, HK633, HK97, mEpX1, mEpX2, and mEp235.

The highest homology was with the phage CUS-3 [15] of the P22 family (a match of 10/18 sequences), followed by HK620 [16] of the P22 family (a match of 8/18 sequences). Eight lambdoid phages also showed a match of 4/18 or 3/18 sequences. Because P22, a phage that infects *Salmonella typhimurium*, is known to share many similarities in genetic structure and regulation with the lambda phage of *E. coli* [17], it is certain that the ECOR52-phage isolated by us is a type of lambdoid phage. Several sequences also showed some similarity with the genomic DNA of other phages or the *E. coli* strains (Table 4), suggesting a mosaic structure of this phage genome. Six sequences (4000L, 4000R, 2400L, 2400R, 1100R, and 7000R; Accession number: AB841352, AB841353, AB841362, AB841363, AB841359, and AB841355) contained non-homologous, unknown sequences (Table 4), each spanning a length of approximately 100–900 bp (total length approximately 2.5 kb), indicating the novelty of this phage.

PCR analysis of the isolated ECOR52-phage DNA was performed using several specific primers (Table 1) designed against the known genomic DNA sequences of 9 phages (CUS-3, HK620, mEp043, HK97, HK022, HK633, HK140, mEpX1, and mEpX2; Table 3). Among the primers tested, amplification was only observed with the primers against the HK140 sequence (data not shown). Two PCR products were detected whose sizes were consistent with those predicted on the basis of the HK140 genome sequence. Therefore, it was demonstrated that the ECOR52-phage possesses a part of the genomic DNA of HK140, a lambdoid coliphage (<http://www.ebi.ac.uk/ena/data/view/JQ086370>).

Taken together, we conclude that the ECOR52-phage is a new P22/lambdoid-like phage closely related to CUS-3, HK620, and HK140. We designated this phage as “MSU52-L1.” There are no previous studies that reported that the ECOR52 strain harbors a lysogenic phage or possesses phage DNA.

4. Conclusion

We identified 34 phage-producing ECOR strains; of them, 14 strains were newly discovered to harbor lysogenic phages. This report is also the first to identify the ECOR52-derived phage as a P22/lambdoid-like phage. Such screening and identification of the lysogenic phages in the ECOR strains should help us understand the genetic background of the ECOR strains and the behavior and functions of accessory DNA in natural *E. coli* strains. Further analysis of the identity of the MSU52-L1 phage and the phages in other ECOR strains is in progress.

Acknowledgements

This work was supported by JSPS KAKENHI Grant Numbers 24650488, 25292051. We are grateful to Prof. Thomas S. Wittam (Michigan State University, USA) for providing the ECOR strains. We would like to thank Enago

(www.enago.jp) for the English language review.

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