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Simple Method for Plating *Escherichia coli* Bacteriophages Forming Very Small Plaques or No Plaques under Standard Conditions▽

Joanna M. Łoś,[1]† Piotr Golec,[1]† Grzegorz Węgrzyn,[1,2] Alicja Węgrzyn,[3] and Marcin Łoś[1]*

Department of Molecular Biology, University of Gdańsk, Kadki 24, 80-822 Gdańsk, Poland[1]; Institute of Oceanology, Polish Academy of Sciences, Powstańców Warszawy 55, 81-721 Sopot, Poland[2]; and Laboratory of Molecular Biology, Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Kladki 24, 80-822 Gdańsk, Poland[3]

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The use of low concentrations (optimally 2.5 to 3.5 μg/ml, depending on top agar thickness) of ampicillin in the bottom agar of the plate allows for formation of highly visible plaques of bacteriophages which otherwise form extremely small plaques or no plaques on *Escherichia coli* lawns. Using this method, we were able to obtain plaques of newly isolated bacteriophages, propagated after induction of prophages present in six *E. coli* O157:H⁻ strains which did not form plaques when standard plating procedures were employed.

In the second half of 20th century, bacteriophages were among the most important models in molecular biology. However, at the end of that century, after learning principles of molecular biology using bacteriophage models, these viruses became considered less attractive research subjects due to development of sophisticated systems for studying eukaryotic cells. Nevertheless, recent years may be recognized as a new era in bacteriophage biology. This is because of several unexpected findings, which demonstrated that viruses infecting bacterial cells may be very important not only in basic biological studies but also in medicine and biotechnology (for reviews, see references 7 and 38). The breakthroughs included discoveries that many toxins (including the vast majority of verotoxins) produced by bacteria pathogenic to animals and humans are encoded in bacteriophage genomes (20, 37); that bacteriophages can be used in vaccination and in specific kinds of therapy (10); that bacteriophages can be extremely dangerous for bioprocesses based on bacterial activities in biotechnological factories (25, 35) but also may be employed in genetic engineering and biotechnology in a very sophisticated manner (10, 28); and finally that they are the most abundant creatures worldwide, thus playing an extremely important ecological role (9, 12, 19, 39).

The growing interest in various aspects of phage biology must be connected to isolation and characterization of newly discovered viruses. However, contrary to well-investigated model bacteriophages, in most cases of newly isolated phages there are problems with their propagation under laboratory conditions. This is partially due to the fact that the number of indicator strains used in laboratories is rather limited and various limitations arise from using nonpermissive or suboptimal hosts. The first step in getting an uncontaminated lysate of a particular bacteriophage strain is obtaining single plaques on the host lawn. However, this first and obligatory stage of analysis may be, in fact, a limiting step as there are many examples (though mostly unpublished) of serious problems with getting plaques of newly discovered bacteriophages. This problem seems to be common in the case of lambdoid bacteriophages (27, 42). We met this problem when investigating phages coding for Shiga toxins, whose genomes are present as prophages in chromosomes of some *Escherichia coli* strains. For example, phage ϕ24B (Δ*stx*::*cat*) (4) and phage ST2-8624 (Δ*stx*::*cat gfp*) from *E. coli* O157:H7 strain 8624 (obtained from Gail Christie, Virginia Commonwealth University, Richmond) form either pinpoint plaques or no plaques, depending on the host strain and particular conditions of standard titration. Therefore, we tried to improve phage titration procedures to develop conditions allowing formation of plaques by bacteriophages which give minute plaques or do not plate at all under standard laboratory conditions.

MATERIALS AND METHODS

Phage and bacterial strains. The *E. coli* strains and bacteriophages used in this work are listed in Table 1.

Bacteriological media. The following media were used. Luria-Bertani (LB) medium (Roth, Germany) (32) was used for bacterial growth. The same medium, but supplemented with 1.5% bacteriological agar, was used as a bottom agar for pouring plates. Top agar consisted of 1% Tryptone (Difco), 0.5% NaCl (POCH, Poland), and 0.7% bacteriological agar (Becton Dickinson). For experiments in minimal media, a phosphate-buffered (FB) mineral salt medium, prepared according to Teich et al. (36) was used. The bottom and top FB agars contained 1.5% and 0.7% bacteriological agar, respectively. The FB mineral medium was prepared using chemicals from POCH, Poland, except for citric acid diammonium salt (Sigma). FB plates were poured using 2× concentrated FB medium mixed with an equal volume of 3% water agar. Top agar consisted of 2× concentrated FB medium mixed with an equal volume of 1.5% water agar. FB media were supplemented with one of following carbon sources: glucose, glycerol, or succinate (all from Sigma) at final concentrations of 0.2%, 0.4%, and 0.6%, respectively.

Antibiotics. All antibiotics (kanamycin, ampicillin, tetracycline, and chloramphenicol [purchased from Sigma]) were tested at the following concentrations: 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5 and 5.0 μg/ml. After preliminary studies, only concentrations that allowed for the formation of the biggest plaques were chosen.

Standard phage titration procedure. Phage titration was performed on disposable plastic petri dishes (diameter, 90 mm) (Merck). Twenty-five milliliters of bottom LB agar plates was used. The indicated amounts of top agar, supplemented with 10 mM $CaCl_2$ (Sigma-Aldrich) and 10 mM $MgSO_4$ (Sigma-Aldrich) and containing the indicated volumes of overnight bacterial culture (according to

* Corresponding author. Mailing address: Department of Molecular Biology, University of Gdańsk, Kładki 24, 80-822 Gdańsk, Poland. Phone: 48 58 5236319. Fax: 48 58 5236424. E-mail: mlos@biotech.ug.gda.pl.

† J.M.L. and P.G. contributed equally to this work.

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TABLE 1. Bacterial strains and bacteriophages used in this work

Bacterial or phage strain	Source or reference
Bacterial strains	
E. coli C600	6
E. coli MG1655	22
E. coli O157:H$^-$ 263	Jacek Osek (Department of Microbiology, National Veterinary Research Institute, Pulawy, Poland)
E. coli O157:H$^-$ 272	Jacek Osek (Department of Microbiology, National Veterinary Research Institute, Pulawy, Poland)
E. coli O157:H$^-$ 282	Jacek Osek (Department of Microbiology, National Veterinary Research Institute, Pulawy, Poland)
E. coli O157:H$^-$ 298	Jacek Osek (Department of Microbiology, National Veterinary Research Institute, Pulawy, Poland)
E. coli O157:H$^-$ 303	Jacek Osek (Department of Microbiology, National Veterinary Research Institute, Pulawy, Poland)
E. coli O157:H$^-$ 306	Jacek Osek (Department of Microbiology, National Veterinary Research Institute, Pulawy, Poland)
Phage strains	
λpapa	Our collection
λ*cIb*2	Our collection
T4D	Our collection
T4rI (r48)	Karin Carlson (Department of Cell and Molecular Biology, Uppsala University, Sweden)
T4rIII (r67)	Karin Carlson (Department of Cell and Molecular Biology, Uppsala University, Sweden)
P1Cm	Hansjoerg Lehnherr (Department for Biochemistry and Molecular Biology, Institute for Microbiology, EMA-University of Greifswald, Germany)
P1vir	Our collection
ϕ24B (Δ*stx*$_2$::*cat*)	4
ST2-8624 (Δ*stx*$_2$::*cat gfp*)	Gail Christie (Virginia Commonwealth University, Richmond)
933W Δtox (Δ*stx*$_2$::*cat*)	16
22Δtox (Δ*stx*$_2$::*cat*)	16
27Δtox (Δ*stx*$_2$::*cat*)	16
32Δtox (Δ*stx*$_2$::*cat*)	16

reference 3, modified to vary the amount of bacterial culture used, as described herein), were poured onto the plate. Five microliters of each serial dilution of a phage lysate was spotted onto the top agar. When full-plate titration was used, 0.1 ml of phage lysate dilutions was added to 1 ml of a bacterial culture (an equivalent of $1.6 \times 10^9 \pm 0.2 \times 10^9$ CFU [mean value from three independent titrations]), which was followed by an addition of 2 or 3 ml of the top agar to the tube and immediate pouring onto the bottom agar layer. Plates were incubated at 37°C for 16 h.

Improved phage titration procedure. The procedure was analogous to the standard phage titration procedure, but the indicated amounts of antibiotics were added to the bottom agar. Top agar remained free of antibiotic at the time of pouring. For a droplet titration variant, top agar was poured immediately before the plates were used.

Plaque size determination. Pictures of plates with bacteriophage plaques were taken using the FluorS (Bio-Rad) gel documentation system. Diameters of plaques were measured manually. A photograph of the scale (in millimeters), taken at the same magnification, was used for calculations.

GT estimation. Generation time (GT) was estimated in liquid cultures by measurement of optical density at 600 nm (OD_{600}). The GT value was calculated from the following equation: $GT = \ln 2/[\ln (OD_{T2}/OD_{T1})/(T2 - T1)]$, where $T1$ and $T2$ are times of measurement in hours. The provided data are mean values from three independent experiments.

RESULTS

When testing different variants of titration conditions, we found that bacteriophages λ*cI*b2 (unable to form lysogens) and T4D (a "wild-type" strain of phage T4) form plaques bigger than those observed on LB media, when titrated on a host (*E. coli* MG1655 [22]) forming a lawn on minimal plates supplemented with a carbon source that supports a rapid (GT of 54.3 ± 1.8 min in a liquid culture, as in the case of glucose), moderate (GT of 81.9 ± 3.6 min in a liquid culture, as in the case of glycerol), or low (GT of 158.2 ± 14.3 min in a liquid culture, as in the case of succinate) growth rate (Fig. 1). However, the effects of reduction of bacterial growth rate using minimal media on enlarging phage plaques were not dramatic. Moreover, in host cells cultured in minimal media, lytic development of at least some bacteriophages, including T4 and λ, is impaired, which is observed as significantly lowered burst size (15, 18, 26). This is also true for T7 phage (see reference 44 and references therein). The lowered burst size does not necessary hamper plaque formation, as this may be partially compensated for by a slower growth of the host. This, in turn, may result in reduction of adsorption rates due to a lower host density increase, facilitating phage spreading by a faster and longer diffusion (2, 5). Knowing that reduction of bacterial growth rate on plates may provide conditions for formation of larger bacteriophage plaques, we aimed to slow the host growth rate down using plates with rich media.

For this purpose, we used relatively low concentrations of antibiotics, which were added to the bottom agar. The top agar layer remained free of an antibiotic, what allowed for its slow diffusion from the bottom layer into the zone of bacterial growth. Various concentrations of four antibiotics (ampicillin, chloramphenicol, tetracycline, and kanamycin) were used. *E. coli* strain C600 (6) was employed in these experiments as a host for phage titration, since in preliminary studies we found

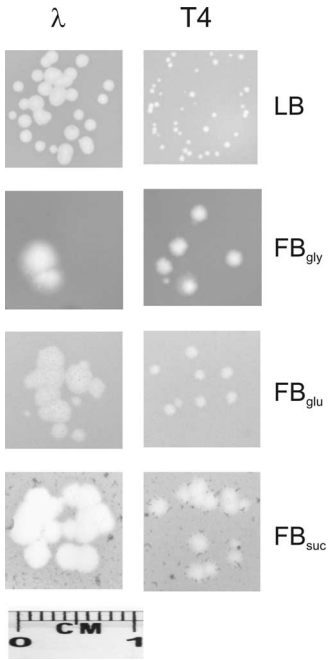

FIG. 1. Formation of plaques by bacteriophages λ*c*Ib2 and T4D on lawns of *E. coli* MG1655 grown in LB medium or minimal FB medium supplemented with various carbon sources (0.4% glycerol, 0.2% glucose, or 0.6% succinate). The size scale is shown.

TABLE 2. Dependence of EOP on amount of overnight bacterial culture and top agar thickness

Amt of overnight *E. coli* C600 culture (μl)	Amt of top agar (ml)	EOP of strain on medium[a]:			
		LB			LB_{cm} (ϕ24B)
		λpapa	T4D	ϕ24B	
20	3	0.36	0.82	$<5 \times 10^{-2}$	1.36
50	3	0.52	1	0.004	2.46
100	3	0.58	1	0.03	3.69
200	3	0.69	0.86	0.23	4.72
400	3	0.79	0.84	0.17	5.56
600	3	0.82	0.65	0.23	6.27
800	3	0.84	0.66	0.56	6.38
1,000	2	1	0.62	1	7.68
1,000	3	1	0.53	0.41	6.43
1,000	4	0.83	0.58	0.61	7.16
1,000	5	0.70	0.31	0.61	5.68

[a] An EOP of 1 corresponds to a maximal number of plaques obtained from the same amount of phage lysate added to the top agar. The EOP of phage ϕ24B plated on LB containing chloramphenicol (LB_{Cm}) is relative to maximal plating efficiency obtained when no antibiotic was used. The values given in the table are relative to EOP (within each phage type) calculated from single replicates of the same lysate, plated out at the same time using the same overnight culture. An EOP of 1 is equivalent to 587, 146, and 503 plaques per plate for λpapa, T4D, and ϕ24B, respectively.

that among standard laboratory strains this one was the most versatile for this purpose (data not shown).

To estimate the effect of the amount of bacterial cultures added to the top agar, we performed the series of experiments presented in Table 2. The efficiency of plating (EOP) of two different lambdoid phages, λ and ϕ24B, was highest when 1 ml of an overnight culture (equivalent of 1.6×10^9 bacterial cells) and 2 ml of the top agar were used. These conditions were then used in testing optimal antibiotic concentrations for improvement of plating of bacteriophages. The concentration finally chosen was a compromise between enlarging plaque size and the ability of bacteria to form a lawn in a top agar. Interestingly, the amount of bacterial culture optimal for bacteriophage T4 plating was about 1 order of magnitude lower than that for λ and ϕ24 (Table 2).

Some significant effects of the tested conditions on formation of plaques by known bacteriophages were observed (Fig. 2). Among all tested antibiotics, only addition of kanamycin resulted in no increase in plaque sizes at any concentrations tested, except for T4 phage, which gave bigger plaques when this antibiotic was employed, and λ phage, which gave bigger plaques, though EOP did fall dramatically. The most significant improvement in plaque sizes was observed on plates with ampicillin at the concentration of 3 μg/ml when 2 ml of the top agar was used and 3.5 μg/ml when 3 ml of the top agar was used (Fig. 2 and Table 3 [data not shown for other antibiotics]). However, in further experiments, we decided to use the concentration of 2.5 μg/ml when 2 ml of the top agar was used, which gave slightly smaller plaques, but revealed significantly higher EOP and reproducibility of the bacterial lawn growth. Plaques formed by phage λpapa on plates with ampicillin were clear (Fig. 2), which might potentially be caused by a lack of lysogen overgrowth, due to an increasing antibiotic concentration in the growth zone of the top agar. This may be also caused by an effect observed by Chakravorty et al. (8), who reported a loss of immunity of spheroplastized *Salmonella* cells to superinfection with a phage for which they were lysogenic. In such a situation, in the final stages of bacterial lawn growth, lysogenic cells, which normally overgrow a center of a plaque, could be spheroplastized and then lysed by a bacteriophage. Addition of either chloramphenicol or tetracycline gave weak results in the case of Shiga toxin-encoding lambdoid phages. Nevertheless, addition of tetracycline improved the size of

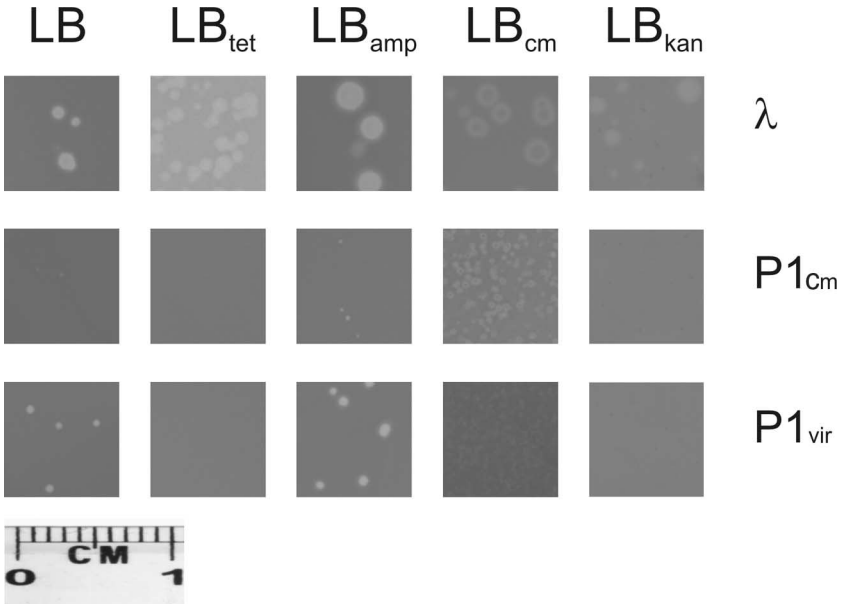

FIG. 2. Formation of plaques by bacteriophages λpapa, P1Cm, and P1vir on lawns of *E. coli* C600 grown in LB medium or in the same medium supplemented with one of the following antibiotics: tetracycline (LB_{tet}), ampicillin (LB_{amp}), chloramphenicol (LB_{cm}), or kanamycin (LB_{kan}). These antibiotics were added to the bottom agar to final concentrations of 1.0, 3.5, 2.5, and 2.5 μg/ml, respectively. The size scale is shown. For plate preparation, 3 ml of the top agar and 1 ml of an overnight bacterial culture (1.6×10^9 CFU) were used.

TABLE 3. Dependence of plaque sizes generated by bacteriophage λ on plates with various ampicillin concentrations and top agar thicknesses

Ampicillin concn (μg/ml)	Mean (SD) % of total plaques within diam range (mm) on[a]:											
	Plates with 2 ml top agar						Plates with 3 ml top agar					
	≤0.5	0.51–1	1.01–1.5	1.51–2	2.01–2.5	≥2.51	≤0.5	0.51–1	1.01–1.5	1.51–2	2.01–2.5	≥2.51
0	41.4 (2.4)	32.9 (0.8)	24.8 (0.4)	0.6 (1.0)	0.0 (0.0)	0.0 (0.0)	38.5 (8.3)	35.9 (7.9)	16.4 (10.1)	6.1 (6.8)	0.0 (0.0)	0.0 (0.0)
0.5	45.8 (0.2)	29.8 (2.6)	22.9 (0.1)	1.1 (1.8)	0.0 (0.0)	0.0 (0.0)	42.5 (0.8)	32.4 (9.0)	18.6 (4.8)	6.6 (3.4)	0.0 (0.0)	0.0 (0.0)
1.0	43.6 (1.0)	25.6 (4.2)	23.4 (2.5)	4.6 (5.3)	0.0 (0.0)	0.0 (0.0)	31.6 (4.1)	30.7 (6.2)	26.8 (0.8)	10.9 (11.1)	0.0 (0.0)	0.0 (0.0)
1.5	28.1 (0.2)	23.3 (0.5)	33.3 (13.6)	14.2 (11.8)	0.7 (1.2)	0.0 (0.0)	31.8 (2.1)	32.4 (5.7)	24.9 (5.0)	10.9 (1.4)	0.2 (0.3)	0.0 (0.0)
2.0	19.8 (0.3)	22.1 (1.6)	39.6 (12.2)	17.5 (12.1)	1.0 (1.4)	0.0 (0.0)	21.1 (3.4)	28.2 (2.3)	23.6 (3.0)	21.6 (1.7)	5.5 (3.7)	0.0 (0.0)
2.5	17.7 (3.6)	31.9 (10.5)	34.8 (1.1)	13.0 (4.2)	2.7 (3.8)	0.0 (0.0)	10.0 (3.9)	11.7 (4.9)	27.5 (1.1)	33.1 (5.7)	16.1 (0.2)	1.5 (2.2)
3.0	12.7 (0.5)	22.3 (7.0)	29.2 (9.4)	25.5 (4.6)	10.4 (2.8)	0.0 (0.0)	7.2 (3.5)	12.4 (5.2)	22.4 (0.3)	29.9 (8.9)	24.9 (1.8)	3.2 (1.6)
3.5	7.9 (0.5)	10.9 (1.4)	18.9 (8.3)	28.9 (11.7)	25.4 (14.8)	7.9 (4.2)	3.3 (4.1)	7.3 (6.6)	11.6 (10.0)	32.2 (20.3)	22.3 (14.7)	12.4 (6.3)
4.0	NA	NA	NA	NA	NA	NA	3.7 (4.4)	11.2 (6.5)	16.0 (5.0)	21.3 (2.6)	30.4 (5.0)	15.6 (8.3)
4.5	NA	NA	NA	NA	NA	NA	3.2 (3.6)	6.3 (5.7)	12.3 (5.4)	15.5 (3.9)	26.3 (2.9)	31.7 (17.7)
5.0	NA	NA	NA	NA	NA	NA	3.4 (3.0)	4.2 (3.7)	5.2 (4.9)	13.7 (1.4)	27.9 (11.9)	39.1 (16.5)

[a] The mean values and standard deviations (SD) are from three experiments (calculated from plates with >100 PFU per plate). NA, not applicable because of lack of growth of a bacterial lawn or incomplete growth of the lawn.

plaques generated by phage λ and both antibiotics showed an ability to improve plaque size generated by phage T4. As these antibiotics lack the ability to spheroplastize cells, one may suggest that the effect caused by ampicillin was a sum of both killing lysogenized cells by superinfecting phage and additional inhibition of growth at later stages of plaque development. Nevertheless, chloramphenicol was particularly effective in increasing EOP and size of plaques generated by phages, which possessed an artificially introduced chloramphenicol resistance gene, namely ϕ24B (Δ*stx*$_2$::*cat*), ST2-8624 (Δ*stx*$_2$::*cat*), 933WΔtox (Δ*stx*$_2$::*cat*), ϕ22Δtox (Δ*stx*$_2$::*cat*), ϕ27Δtox (Δ*stx*$_2$::*cat*), and ϕ32Δtox (Δ*stx*$_2$::*cat*) (Fig. 3 and 4 and Table 4), and to some extent P1Cm. This may be explained by an effect of a chloramphenicol resistance gene present in genomes of all these

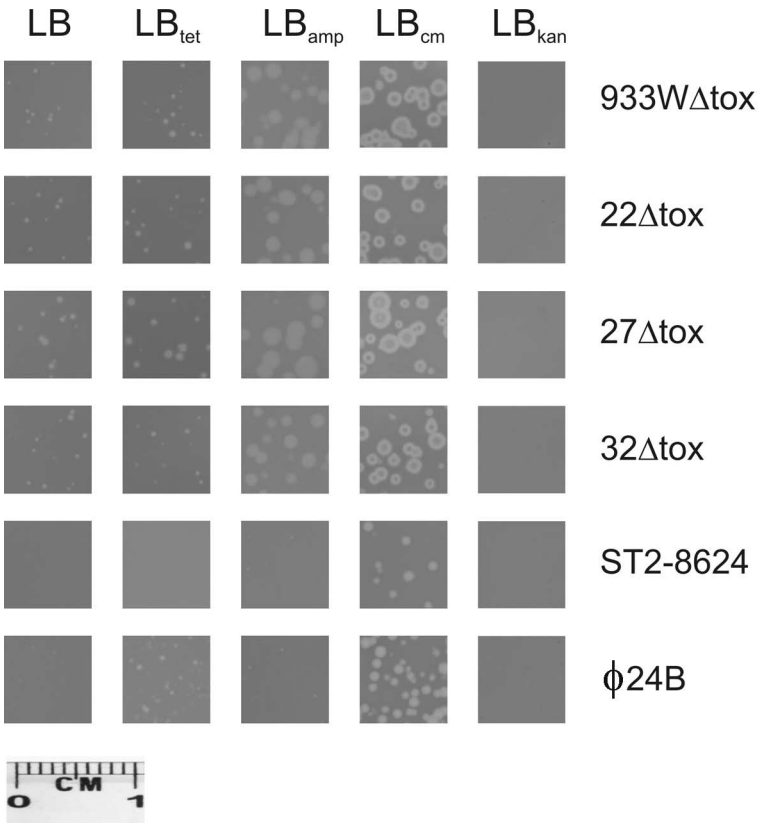

FIG. 3. Formation of plaques of bacteriophages 933WΔtox (Δ*stx*$_2$::*cat gfp*), 22Δtox (Δ*stx*$_2$::*cat gfp*), 27Δtox (Δ*stx*$_2$::*cat gfp*), 32Δtox (Δ*stx*$_2$::*cat gfp*), ST2-8624 (Δ*stx*$_2$::*cat gfp*), and ϕ24B (Δ*stx*$_2$::*cat*) on lawns of *E. coli* C600 growing in the LB medium or in the same medium supplemented with one of the following antibiotics: tetracycline (LB$_{tet}$), ampicillin (LB$_{amp}$), or chloramphenicol (LB$_{cm}$). These antibiotics were added to the bottom agar to final concentrations of 1.0, 3.5, and 2.5 μg/ml, respectively. The size scale is shown. For plate preparation, 3 ml of the top agar and 1 ml of an overnight bacterial culture (1.6×10^9 CFU) were used.

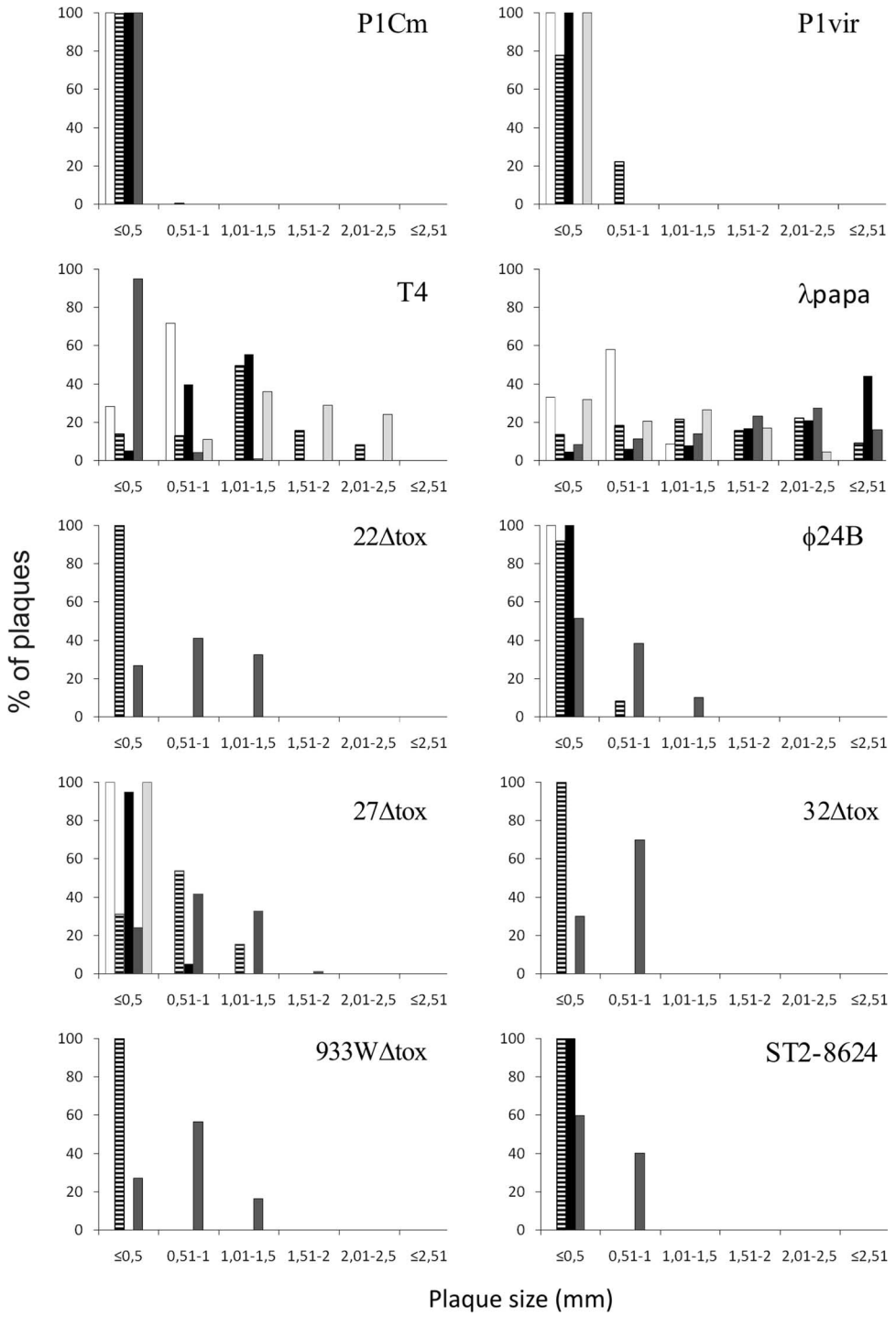

FIG. 4. Changes in fractions of plaques of given diameter, generated by indicated bacteriophages on *E. coli* C600 grown on media containing the following antibiotics: none (open bars), 2.5 μg/ml ampicillin (bars with horizontal stripes), 1 μg/ml tetracycline (black bars), 2.5 μg/ml chloramphenicol (gray bars), and 2.5 μg/ml kanamycin (light gray bars). The absence of a bar indicates a lack of plaques. The results of each experiment are based on measurement of 100 to 300 plaques. Note also the information about EOP (Table 4).

phages, whose expression after infection of the host may increase cellular productivity by alleviating inhibitory effects of the antibiotic on protein production, specifically in infected cells. In the case of other phages, the main effect might be assumed to be caused by a slower growth of bacterial lawns: however, tetracycline was effective in increasing the sizes of plaques generated by lambdoid phages and T4 but was ineffective in the case of P1Cm. Moreover, the dependence of efficiency of plating on the kind of antibiotic added to the bottom agar, which was significant for lambdoid bacterio-

TABLE 4. EOP of various bacteriophages using different antibiotics

Phage	EOP on medium[a]:				
	LB	LB_{cm}	LB_{amp}	LB_{tet}	LB_{kan}
λ	1.00 ± 0.07	0.36 ± 0.012	0.48 ± 0.12	0.56 ± 0.019	0.035 ± 0.028
ϕ24b	0.048 ± 0.016	1.00 ± 0.11	0.22 ± 0.051	0.22 ± 0.058	$<10^{-3}$
22Δtox	$<10^{-3}$	1.00 ± 0.11	0.16 ± 0.044	0.0077 ± 0.0058	$<10^{-3}$
27Δtox	0.25 ± 0.02	1.00 ± 0.14	0.49 ± 0.08	0.36 ± 0.21	$<10^{-3}$
32Δtox	0.017 ± 0.003	1.00 ± 0.16	0.098 ± 0.11	$<10^{-3}$	$<10^{-3}$
933WΔtox	$<10^{-3}$	1.00 ± 0.17	0.20 ± 0.09	$<10^{-3}$	$<10^{-3}$
ST2-8624	$<10^{-3}$	1.00 ± 0.077	0.49 ± 0.15	0.078 ± 0.011	$<10^{-3}$
P1vir	0.64 ± 0.1	0.09 ± 0.07	1.00 ± 0.07	0.14 ± 0.027	0.31 ± 0.06
P1Cm	0.49 ± 0.07	0.026 ± 0.019	1.00 ± 0.07	$<10^{-3}$	$<10^{-3}$

[a] The estimated EOP is relative to an average result obtained under conditions of the highest EOP. The sensitivity of $<10^{-3}$ means that no plaques were observed under the given conditions. LB medium antibiotic abbreviations: cm, chloramphenicol; amp, ampicillin; tet, tetracycline; kan, kanamycin. The results presented are average values from at least three independent experiments ± standard deviation.

phages and P1Cm (Table 4), was not observed for T4 phage, which plated equally well irrespective of the presence of any antibiotic (data not shown).

To test the efficiency of the described method in isolation of bacteriophages de novo, we tested six *E. coli* O157:H$^-$ strains (obtained from Jacek Osek, Department of Microbiology, National Veterinary Research Institute, Pulawy, Poland). These strains, cultured in LB medium, were treated with mitomycin C (final concentration of 0.5 μg/ml), and cultivation was prolonged to allow propagation of induced phages and liberation of phage progeny. In the analysis of obtained lysates, we were able to detect plaques formed by the phages only on plates with ampicillin added to the bottom agar (Fig. 5). We failed to detect plaques of these phages (using the same lysates) on standard LB plates and on plates with other antibiotics. Moreover, no plaques of these phages were formed on bacterial lawns grown on plates with minimal media (data not shown). It is also worth mentioning that the effects of antibiotics were significantly more pronounced than the effects of minimal media in the case of all tested phages (data not shown).

DISCUSSION

Bacteriophage plaque formation is a process which is not fully understood yet. Although relatively many factors may influence this process, only some of them are relatively well studied. Thus, problems with titration of many phages, including newly isolated strains, are quite common in the laboratory practice. The use of antibiotics for improvement of plating efficiency has already been proposed by Yin et al. (41); however, they added antibiotics to kill bacteria contaminating the sample. Moreover, bacteria used for plating were resistant to the antibiotic used; thus, the effect was not the plating improvement itself, but rather uncovering plaques which would be masked by colonies of contaminating bacteria. Here, we describe a modification of the titration procedure allowing us to obtain relatively large plaques of phages which otherwise give very small plaques or no plaques. The modification is based on the use of low concentrations of antibiotics in the bottom agar. An increase in the size of plaques, caused by low concentrations of antibiotics in the bottom agar, might be explained by the plaque growth kinetics, which is limited mostly by a high host cell density. This is due to an increasing

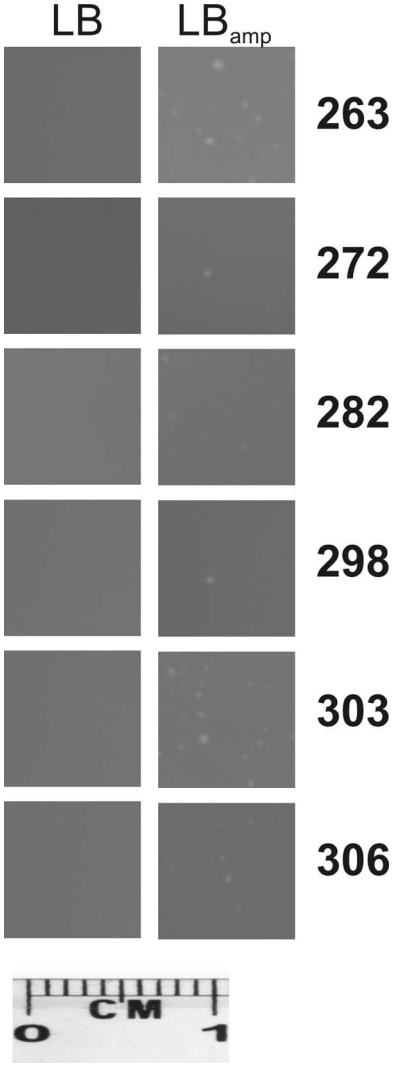

FIG. 5. Formation of plaques of bacteriophages induced by mitomycin C treatment (0.5 μg/ml) from *E. coli* O157:H$^-$ strains 263, 272, 282, 298, 303, and 306 on lawns of *E. coli* C600 grown in LB medium or LB medium supplemented with ampicillin (LB_{amp}) to a final concentration of 3.5 μg/ml. The size scale is shown.

depletion of virus particles by their adsorption on host cells and fragments of lysed cells and by a physical blocking of diffusion of phages, independently of the adsorption kinetics (5, 14, 43). This effect can be partially overcome by using media supporting slow growth of bacterial cells. However, the use of media supporting slow bacterial growth may lead to a lowering of the burst size of infected cells (23, 44). Considerably better results obtained with the use of low ampicillin concentrations, relative to other antibiotics, could be explained by the effect of this antibiotic on bacterial cells and, in turn, on phage burst size. At low concentrations, ampicillin, like other β-lactam antibiotics, causes an increase in the size of bacterial cells by inhibiting the cell division process without decreasing the growth of the cell mass (17). As determined previously (18), bigger cells give larger phage burst sizes: thus, ampicillin-treated (but still growing) cells can produce more virions. This effect of ampicillin on phage growth may be transient as bacteria spheroplastized by higher concentration of penicillin show smaller burst size (31) and bacteria lysogenized by superinfecting phage may lose immunity to superinfection (8). Moreover, at later stages of plaque formation, when antibiotic concentration in the bacterial growth zone increases, ampicillin may act synergistically to muraminolytic activities, which are associated with some phage virions (29). This might partially explain the formation of bigger plaques by phage T4D, as even slight damage of the bacterial cell wall, mediated by ampicillin, may trigger a lysis inhibition collapse during plaque formation (1). The increase of plaque size observed on plates with kanamycin, and to some extent with tetracycline, may be an effect of a partial imbalance in the regulation of lysis inhibition in the host with an impaired, but not fully suppressed, protein synthesis system. However, in such a situation, a lack of plaque size enlargement by addition of chloramphenicol suggests that the nature of antibiotic action, not only its final effect, may play a role in the modulation of plaque formation by bacteriophages.

While the manuscript for this article was being revised, a work by Comerau et al. (11) was published. Results reported in that article may facilitate better understanding of our results. The observation of those authors that β-lactam and quinolone antibiotics cause an increase in plaque sizes when used at concentrations sublethal for bacterial hosts was explained partially by an increase in phage burst size under such conditions. Earlier work (40) showed that cell filamentation, especially when caused by mitomycin C and UV light, had a similar impact on T4 phage plaques. However, the effect was not linked to the SOS response, which is triggered by all of these antibiotics and UV irradiation, but rather to filamentation of bacterial cells (see reference 11 and references therein).

Differences in susceptibility of plaque sizes to different factors (showed in this work), like the amount of bacteria in the top agar and the kind of antibiotic used in the bottom agar, suggest that the processes of plaque formation by different groups of bacteriophages progress in different manners and may depend on different factors. Ampicillin was shown to be the most versatile agent, improving plating efficiency of lambdoid bacteriophages and both variants of P1. Addition of chloramphenicol gave outstanding results in all lambdoid bacteriophages with the chloramphenicol resistance cassette inserted into their genomes. Very poor plating efficiency of P1Cm on plates with chloramphenicol is a surprising result, which shows that plaque formation by this phage is dependent on different factors from that of lambdoid phages.

During isolation of phages from environmental sources, bacteria which contaminate the samples may inhibit plaques' formation by simply overgrowing a bacterial lawn. It was suggested by Yin et al. (41) that one might use an antibiotic-resistant strain and media containing this antibiotic to kill the contaminating bacteria. Our results may suggest that environmental phagologists could additionally use sublethal concentrations of other antibiotics to which bacteria forming a lawn are not resistant. This may result in an increase in plaque diameters and isolation of bacteriophages which otherwise would be overlooked due to formation of small plaques or no plaques. This approach may also reduce the necessity to concentrate samples to obtain the proper number of phages which plate with very small efficiencies. An alternative method for plaque size increase (24), and thus for de novo isolation of phages, is addition of glycine to a *Lactobacillus* lawn (13). However, this method does not work for *E. coli* phages, as glycine does not slow *E. coli* growth (data not shown). When a material from environmental samples is used for isolation of phages which do not plate efficiently or do not plate at all, replacement of top agar with 0.2% agarose in two-layer plates may facilitate phages to develop normal-size plaques (21, 33, 34). One could expect that the mechanisms of plaque enlargement by sublethal antibiotic concentrations and by the use of lowered concentration of the top agar are different, and thus they may act synergistically. As wide spectra of antibiotics can affect many bacterial species, they can potentially be used for isolation of bacteriophages other than coliphages. Thus, the method developed here may improve the ability to isolate pure phage cultures from habitats where only metagenomic approaches have been used to date.

The ability to improve plating and plaque visibility of coliphages may also be used in environmental approaches like, e.g., water quality control. One of the methods which allows for detection of fecal contamination of water is estimation of coliphage load in water samples (30). The use of the method presented in this report may allow for higher sensitivity of detection of bacteriophages.

In summary, in the case of plating of phages which form extremely small plaques or from which obtaining plaques is very difficult (or even impossible) under standard conditions, we propose to use plates supplemented with 2.5 μg/ml ampicillin in the bottom agar. When this approach fails, or when it is impossible to use this antibiotic due to bacterial resistance, other antibiotics should be tested. When an investigated phage bears in its genome a gene responsible for resistance to another antibiotic, the use of this antibiotic may also be recommended.

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