A growing microcolony can survive and support persistent propagation of virulent phages

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Edited by Bruce R. Levin, Emory University, Atlanta, GA, and approved November 27, 2017 (received for review May 30, 2017)

Bacteria form colonies and secrete extracellular polymeric substances that surround the individual cells. These spatial structures are often associated with collaboration and quorum sensing between the bacteria. Here we investigate the mutual protection provided by spherical growth of a monoclonal colony during exposure to phages that proliferate on its surface. As a proof of concept we exposed growing colonies of *Escherichia coli* to a virulent mutant of phage P1. When the colony consists of less than ~50,000 members it is eliminated, while larger initial colonies allow long-term survival of both phage-resistant mutants and, importantly, colonies of mostly phage-sensitive members. A mathematical model predicts that colonies formed solely by phage-sensitive bacteria can survive because the growth of bacteria throughout the colony exceeds the killing of bacteria on the surface and pinpoints how the critical colony size depends on key parameters in the phage infection cycle.

bacteria | spatial structure | coexistence | endemic | predator-prey

Virulent phages are ubiquitous, yet seemingly at odds with their own survival. As they kill their susceptible hosts, they suppress these to low population levels (1–3), creating a fragile and hugely competitive environment for themselves (4–6). By contrast, temperate phages have the option to lysogenize their host and thereby preserve their DNA through periods of low host availability (7, 8). This apparent advantage of temperate over virulent phages suggests the need for a more fine-grained understanding of virulence.

Notably, the virulent phage species present a quite diverse set of characteristics for their killing. Even under identical conditions, different species have average burst sizes that vary by more than a factor of 10 and have latency times that vary by a factor of 4 (9). Some virulent phages such as T3 and T7 are known to form particularly large plaques (10), while for example T4 forms rather small plaques due to lysis inhibition (10, 11), reflecting the variation in the “art of killing” (12).

Here we explore a demographic aspect of virulence, namely how a virulent phage propagates in a growing bacterial colony. Our investigation is inspired by visual inspection of the plaque formed by a virulent phage in Fig. 1. The picture shows a “clear” plaque, caused by one P1_{vir} phage that was introduced onto a bacterial lawn consisting of *Escherichia coli* cells. A nearly clear region surrounds the initial infection at the center, but one can also observe increasingly larger faint colonies as one moves away from this center. Outside the plaque, the agar contains a dense collection of colonies that have grown to stationary phase without the influence of phages. Bacteria in the periphery of the plaque encountered phages relatively later, after each bacterial cell had grown to form a microcolony (13). The origin of the faint colonies at the plaque periphery has not been fully understood (14).

This paper explores the fate of growing bacterial colonies that are exposed to phages at various time points during their growth. A previously proposed scenario (14) discussed that cells in the center of a large microcolony may enter stationary phase and become unable to support the production of progeny phages.

Although such a protection mechanism is certainly expected, recent work showed that the growth of even quite large *E. coli* colonies (~10⁷ cells) mimics the normal exponential growth of individual *E. coli* cells in liquid medium (15). This finding indicates that nutrients diffuse extensively through the colony. By contrast, phages may well adsorb in large numbers to susceptible cells on the colony surface and therefore only rarely reach deeper layers of the colony. Thus, we hypothesize that the continued growth from the inside of a sufficiently large colony could overwhelm killing on the colony surface. In combination with the phage-refractory state of stationary-phase cells, the result would be long-term survival of phage-sensitive cells inside the colony.

Here, we first construct a model of growing cells and infecting phages to numerically simulate the phage attack on a growing microcolony. The simulation suggests a sharp transition from elimination of the microcolony to its continued growth as the microcolony size at the time of the first phage encounter increases. We then show that the model prediction is consistent with an experiment by infecting *E. coli* colonies of different sizes with P1_{vir} and monitoring their fate. Our study demonstrates that a sufficiently large colony indeed provides a refuge against P1_{vir}. The refuge counteracts the phages’ ability to control bacterial biomass (6) and thereby allows the bacteria to circumvent the famous kill-the-winner (16) feature of phage predation in well-mixed environments.

Results

Model. We simulate microcolony growth by modeling each cell as a sphere, and each cell grows and divides exponentially with a minimum doubling time of ~20 min. The repulsive force

Significance

Bacteria are repeatedly exposed to an excess of phages and carry evidence of this in terms of multiple defense mechanisms encoded in their genome. In addition to molecular mechanisms, bacteria may exploit the defense of spatial refuges. Here we demonstrate how bacteria can limit the impact of a virulent phage attack by growing as a colony which exposes only its surface to phages. We identify a critical size of the initial colony, below which the phages entirely eliminate the colony and above which the colony continues to grow despite the presence of phages. Our study suggests that coexistence of phages and bacteria is strongly influenced by the spatial composition of microcolonies of susceptible bacteria.

Author contributions: R.S.E., S.L.S., K.S., and N.M. designed research, performed research, analyzed data, and wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1708954115/-DCSupplemental.

Fig. 1. Plaque formed by P1_{vir}. Green spheres are microcolonies of *E. coli* expressing green fluorescent protein. Note the faint colonies, which are seen across the plaque although less in its central region. (Scale bar: 300 μm .)

between the cells provides an overall spherical growth of the microcolony consisting of densely packed bacteria. At a certain time, we let the surface of a grown microcolony be surrounded by $\sim 2,700$ phages and observe whether the phages kill all the cells or whether the cells can outgrow the ongoing lysis by the phages. The simulated microcolonies are rather small, and the explicit modeling of nutrient gradients inside a microcolony gave only minor growth rate differences across the colony (*SI Appendix, section 3*). Therefore, we here present the results from simulations that assume a constant nutrient level across the microcolony. The detailed simulation protocol is described in *Methods* and *SI Appendix, section 1*, with the default parameter values with dimensions (mostly measured in liquid culture conditions) in *Table S1*.

Fig. 2 illustrates model simulations with uninfected bacterial cells shown as blue spheres. Bacteria infected by phages are colored from yellow to red, where the ones closest to lysis are assigned the most reddish color. Each lysis event is modeled by replacement of the bacterium with 400 point-like phage particles at the end of the latency period. The progeny phages (not shown in the image) are allowed to diffuse and infect other bacteria. In this simulation, we assume that a phage particle adsorbs to a cell as soon as they overlap, mimicking the diffusion-limited scenario of adsorption. The phages adsorb equally well to uninfected cells and those that are already infected by another phage(s). We later study the effect of a smaller adsorption rate on microcolony growth.

In Fig. 2, *Upper row*, the first attack occurs at 3.25 h of growth, when the colony has a size of ~ 220 members. Fig. 2, *Lower row* depicts a phage attack at 3.75 h where the colony size has reached ~ 500 members. The smaller colony is eventually entirely eliminated, which would leave the fate of the phage particles to the chance that they reach another colony before they decay. In contrast, when the phages attack the larger colony, it survives and grows with phages persisting on the infected surface. The simulation showed that the distance phages can penetrate into the microcolony, ΔR , is roughly constant over time when the microcolony size is above the threshold of killing (*SI Appendix, section 4*). For the parameters used in Fig. 2, phages can infect about two layers of cells. This limited penetration is observed because new phages released by lysed cells on the surface mostly adsorb to the cells in their immediate vicinity instead of diffusing deeper into the microcolony. The result is a high multiplicity of infection of the surface cells (cf.

ref. 17) with the cells closer to bursting located nearer to the surface (Fig. 2) and preservation of uninfected cells closer to the center.

Fig. 3 examines the predictions of the model. Fig. 3*A* shows the final growth rate of a colony as a function of the time at which it first encountered the phages. Indeed, the model predicts a sharp transition from extinction to persistent growth, when the size of the microcolony has surpassed a lower critical threshold. It is also apparent that if the first infection occurs sufficiently late to allow colony survival, the colony will reach a size where the growth penalty associated with infections at its surface becomes relatively small. Note that the growth rate is measured while the nutrient depletion is assumed to be negligible. In reality this assumption will ultimately break down when the inner part of the colony becomes increasingly shielded from available nutrients. The lack of nutrients will, however, also prevent or at least reduce phage proliferation (10, 14). Thus, if the colony is above the survival threshold, it is predicted to eventually maintain a large number of living sensitive cells in the center.

Assuming a constant penetration depth ΔR of the phages into a growing colony allows us to understand the steep size-dependent transition in microcolony fate. Suppose that a spherical microcolony of radius $R(t)$ at time t consists of an inner core of exponentially growing uninfected cells with growth rate of biomass g and an infected surface layer of a constant thickness ΔR . We further assume that infected cells will burst with a rate $1/\tau_L$, with τ_L being the latency time of the phage burst. This leads to the equation for the total volume $V(t) = \frac{4\pi}{3} R(t)^3$ to be

$$\frac{dV(t)}{dt} = \frac{4}{3}\pi \left[g(R(t) - \Delta R)^3 - \frac{1}{\tau_L} [R(t)^3 - (R(t) - \Delta R)^3] \right],$$

where the first term in the brackets depicts the growth of the uninfected cells in the inner core, while the second term describes the lysis of the outer infected layer. Clearly, the growth of the total volume $dV(t)/dt > 0$ requires large enough $R(t)$ compared with ΔR . The critical threshold R_c for the initial size $R(0)$ is determined by $dV(0)/dt = 0$. The solution is given in *SI Appendix, Eq. S7* and fits well with the simulation (Fig. 3*B*). If we approximate the solution for a small penetration depth case ($\Delta R \ll R_c$), we get $R_c = 3[1 + (g\tau_L)^{-1}]\Delta R$ (derivation in *SI Appendix, section 5*), depicting that R_c increases with ΔR and that a smaller burst rate $1/\tau_L$ compared with the cell growth rate g decreases R_c .

R_c depends on the various parameters as demonstrated in Fig. 3*B–D*. R_c grows roughly linearly with $1/\tau_L$ (Fig. 3*B*), because the latency time defines the timespan where an infected cell can

Fig. 2. Snapshots of simulations with half of the microcolonies presented. (*Upper row*) The case of a relatively early phage attack (infection at 3.25 h), which results in elimination of the microcolony. (*Lower row*) The history of a slightly later attack (infection at 3.75 h), which results in continued growth of the microcolony.

Fig. 3. Model predictions. (A) Long-term growth rate of phage-infected colony as a function of initial exposure time (T_i). The effective growth rate g was determined by fitting the analytical solution (SI Appendix, Eq. S9) of the linearized version of the model equation to the simulation time traces of the radius $R(t)$ (see SI Appendix, section 5 for details). The radius of the colony at the initial exposure time is shown on the top horizontal axis. (B) The critical radius R_c as a function of latency time τ_L for burst sizes of 100 (open blue circles), 400 (red crosses), and 1,000 (open black triangles). The fit of SI Appendix, Eq. S7 to each data point is shown as a solid line, with the fitting parameter $\Delta R = 1.26 \mu\text{m}$, $1.40 \mu\text{m}$, and $1.53 \mu\text{m}$, respectively. (C) R_c as a function of the phage diffusion constant D_p . (D) R_c as a function of the phage adsorption rate η . D, Inset shows the phage penetration depth ΔR . The SEM is shown as error bars.

continue to shield the uninfected core by adsorbing additional phage particles until it undergoes lysis. The burst size affects ΔR and hence R_c , but changing the burst size from 100 to 1,000 increases the critical radius R_c only by about one cell layer with the given parameter set (Fig. 3B). A larger phage diffusion constant D_p only marginally increases the critical radius (Fig. 3C), because the phages are hindered from reaching the core cells by adsorption to the infected surface cells regardless of the magnitude of the diffusion constant.

Reducing the adsorption rate η from the diffusion-limited case causes a substantial increase in the critical radius R_c . To simulate small η we allowed the phage particles that overlap a cell to be repelled away and introduced a finite rate γ with which infection can occur while there is overlap. Reducing γ parameterizes a reduction of the phage adsorption rate η from the diffusion-limited value. The reduction of η dramatically increases the critical radius R_c (Fig. 3D). Note that the values of η shown are determined in the well-mixed condition (SI Appendix, section 2), and the actual adsorption events to densely packed hosts cannot be simply given by η multiplied by the densities of the phage and bacteria. The reduction of R_c is caused by an increased penetration depth ΔR as phages can diffuse deeper into the colony core before they adsorb to a cell, demonstrating the importance of the high multiplicity of infection of cells at the surface for the protection of the core.

Infection of *E. coli* Colonies with $P1_{vir}$. To test the above predictions in the laboratory, we performed infections of *E. coli* microcolonies with a virulent mutant of the phage P1, $P1_{vir}$

(18). This phage has a fairly long latency time of 60 min for *E. coli* growing in rich medium (9), thus facilitating a moderately small value of R_c . As the bacterial host, we used *E. coli* strain SP427 (19), which expresses green fluorescent protein (GFP) constitutively. The GFP expression allowed us to visualize the intact cells in the colonies using fluorescence microscopy. We grew the host cells embedded in a thin soft-agar layer on plates containing rich medium, which prevents the cells from swimming and causes them to form compact, approximately spherical colonies. We then sprayed phage lysate onto these plates at different times after incubation, thereby implementing phage attacks on colonies of different sizes (Methods). We sprayed enough phages that all of the phage-sensitive cells would have been killed if the same phage-to-cell ratio had been mixed in a homogeneous liquid culture (details in SI Appendix, section 9).

Fig. 4 shows images of representative colonies at the time of addition of phage $P1_{vir}$ and at 16 h of incubation after phage addition. Fig. 4, Left shows the dark-field images, whereas Fig. 4, Right shows the green fluorescence images, which visualize intact bacteria that contain GFP. The panel “5h” displays the rather small colony size that was typically obtained after 5 h of incubation. The “5h+16h” panel (Fig. 4, Top) shows the typical final colony after an additional 16 h of incubation without exposure to phage. In this case, the colony is not only large

Fig. 4. Images of typical colonies before and after exposure to phages. The dark-field image and the corresponding green fluorescence image of the same colony are shown side by side. The green fluorescence images in the final colonies with phage exposure at 5 h and at 7 h spray time were collected at the same light source strength and with the same exposure time and are shown in the same magnification to allow direct comparison. Images of colonies that experienced different phage exposure times but identical total incubation times are shown in SI Appendix, Figs. S9 and S10.

Fig. 5. $P1_{vir}$ infection of *E. coli* microcolonies. (A) Growth in colony radius over time in the absence of phages. The solid line marks an exponential fit with a doubling time of 31 min. The dashed line is a linear fit to growth in colony radius on longer timescales. The crossover point is ~ 10 h. The radius was estimated from the colony image from the area A as $\sqrt{A/\pi}$. (B) Final colony size as a function of the incubation time before phage exposure. The solid red symbols show the estimates from dark-field images whereas the open green symbols show the radius estimated from the green fluorescence images. The triangles between 200 μm and 400 μm show the final radius of colonies in the absence of phages, while the circles show the final radius of colonies exposed to phages. The SEM is shown as error bars. (C) Number of visible colonies on plates sprayed with phages relative to the mean number of colonies on control plates without phages. The control plates had 76 ± 4 visible colonies per plate. The horizontal axis shows the microcolony growth time before the exposure to phages.

but also dense and homogeneously fluorescent. In contrast, the “5h+16h phage” panel (Fig. 4, *Upper Middle*) shows a typical final colony when the plate has been sprayed with phage lysate after 5 h of growth and then incubated 16 h after that. One sees that the colony has grown bigger after incubation in the presence of phages, but it exhibits only faint fluorescence, indicating that most of the colony shown in the dark-field image consisted of dead remnants of bacteria.

The typical colony obtained after 7 h undisturbed growth is shown in the “7h” panel in Fig. 4, *Bottom*, and is visibly larger than the typical colony after 5 h of incubation, reflecting the additional four doublings of biomass. The panel “7h+16h phage” shows the final size of this colony after it was exposed to phages and then incubated for 16 more hours. Although it is visibly smaller than the undisturbed colony in the “7h+16h” panel, one observes a fluorescent region in the middle, suggesting a substantial amount of surviving bacteria.

Using microscopy images, we measured the growth of colonies that had not been exposed to phages and show that the radius grows exponentially (Fig. 5A). This growth is consistent with a doubling time of 30 min for about 9 h until a size of 2×10^6 bacteria if we assume a spherical colony of dense cells with volumes of $1 \mu\text{m}^3$. After about 10 h, the growth rate is reduced substantially and colony size eventually stabilizes at a level set by the initially available nutrients and the number of colonies per plate sharing those nutrients. Fig. 5B shows how this final colony size varies moderately between experiments without phage exposure (triangles). The final radius of 400 μm implies that our conditions allow colonies to reach $\sim 2 \times 10^8$ bacteria. Because slow-growing bacteria are smaller than rapidly growing bacteria (20), we cannot accurately deduce the actual number of bacteria in these colonies.

Predictably, exposure to phages alters the colonies dramatically. Fig. 5B (circles) shows the final size of colonies that were exposed to phages. In the dark-field recording we see a moderate change in the radius of final colonies from about 90 μm to about 180 μm as the phage exposure time is delayed. The corresponding change in size of the GFP-expressing part of the colony increases more dramatically from 20 μm to about 100 μm . In all cases, the outer layers of the colony appear dark, indicating that the surfaces of these colonies consist of bacterial debris from *E. coli* that were killed by phages. Fig. 5C quantifies the number of colonies per plate that are visible with the naked eye after the final incubation period for different prephage incubation times. The surviving fraction is normalized to the average number of visible colonies per control plate that were not exposed to phage. Almost no colonies had grown to visible size when the phages were introduced earlier than 4 h of incubation. The absence of surviving colonies was confirmed using fluorescence microscopy with $10\times$ magnification. After this critical time, visible colonies emerged. When phages were introduced at 4 h, a few small colonies could be detected. When phages were introduced at 6 h and later, almost 100% of the initial cells grew to form visible colonies. Thus, this laboratory experiment reproduced a steep transition in the number of surviving colonies that depended on the size of the microcolony at the time of phage addition. Two additional repetitions of the experiment confirmed the jump in colony survival when phages were introduced after 5–6 h of prephage incubation (*SI Appendix, section 6*).

An alternative explanation for the microcolony size-dependent jump in colony survival (Fig. 5C) could be that the jump reflects the critical colony size at which there is a large probability that the colony contains one or more mutated cells that have become phage resistant at the time of phage exposure and therefore can continue to grow and form a colony despite the killing of all of the phage-sensitive cells. Phage resistance and our spatial refuge model are not mutually exclusive but could both contribute to colony survival. To estimate the frequency of resistant bacteria in the surviving microcolonies, we picked cells from 50 of the microcolonies exposed to phages and restreaked them onto fresh plates to obtain “offspring” colonies formed by individual surviving cells in the presence of the chelator sodium citrate, which inactivates free phages and thereby permits colony formation by both sensitive and resistant cells. Up to 10 randomly picked offspring colonies from each original microcolony were then tested for resistance to $P1_{vir}$ by cross-streaking (see *SI Appendix, Table S2* for complete results).

Table 1, “offspring,” shows that sensitive and resistant cells were found in approximately equal numbers among the randomly picked offspring colonies arising from microcolonies that had been sprayed with phage after 4–8 h of prephage incubation. While these numbers demonstrate the expected strong selection pressure favoring phage-resistant mutants, which increased in frequency from 1.2×10^{-5} in the starting culture to ~ 0.5 in the surviving colonies, they also demonstrate that many phage-sensitive cells can survive the attack by $P1_{vir}$. Importantly, when the offspring are grouped by the microcolony they originated

Table 1. $P1_{vir}$ resistance test summary

Time, h	“Offspring”			Original microcolonies			
	Sensitive	Resistant	Total	All sensitive	Mix	All resistant	Total
4	47	13	60	4	1	1	6
5	22	10	32	4	0	1	5
6	20	50	70	2	0	5	7
7	41	45	86	4	1	4	9
8	51	49	100	5	1	4	10
Total	181	167	348	19	3	15	37

overlaps with a cell, a repulsive force is exerted from the cell. A phage overlapping with a cell is adsorbed with a rate γ . When a cell is infected by a phage, it produces phage particles after an average latency time τ_L (13). During the latency period, an infected cell can adsorb more phages. When a lysis occurs, β new phage particles are spawned uniformly around the cell's center and the cell is removed. The simulation starts with a single cell, which is allowed to establish a colony for the time duration T_i . At time T_i , phages are spawned uniformly in the simulated volume outside of the colony. Details are in [SI Appendix, section 1](#). The code is available as [Dataset S1](#).

Infection of *E. coli* Microcolonies with P1_{vir}. *E. coli* strain SP427 (19), an MC4100 derivative containing a chromosomally encoded $P_{A1/O4/O3}::gfpmut3b$ gene cassette (32), was grown overnight in LB (33) supplemented with 50 μ g/mL kanamycin. The culture was diluted to $\sim$ 400 cfu/mL in sterile MC buffer (50 mM CaCl₂, 25 mM MgCl₂) and kept at room temperature. Every hour, 250 μ L of the diluted culture was mixed with 2.5 mL R-top agar (as in ref. 34 but with 5 g/L agar and 50 μ g/mL kanamycin), plated on fresh R agar plates (as R top but with 12 g/L agar), and incubated at 37 °C to allow individual cells to form microcolonies. After a given incubation

time at 37 °C, 0.5 mL of P1_{vir} phage lysate (7×10^9 pfu/mL) was sprayed uniformly on the top agar surface, using a perfume bottle with an atomizer bulb (35), and the plate was incubated for another 16 h at 37 °C. One control plate without the addition of P1_{vir} was processed along with triplicate phage-spray plates for each time point. The experiment was repeated three times with similar results. Details of the repeat experiments can be found in [SI Appendix, section 6](#). Microscopy images were obtained using a Leica MZ16F fluorescence stereomicroscope and quantification was done by matlab code developed by the authors. The peripheral 1 cm of each plate was excluded in the analysis to avoid any effects of heterogeneity in phage spraying close to the edge. Six additional plates without phage addition were prepared as the control plates above and used for microscopy imaging to determine microcolony size from 5–10 h of incubation at 37 °C.

ACKNOWLEDGMENTS. The authors sincerely thank Stanley Brown for helpful discussions and Linda Hove Christensen for technical assistance. This work was funded by the Danish National Research Foundation (BASP: DNRF120) and the European Research Council under the European Union's Seventh Framework Programme (FP/2007 2013)/ERC Grant Agreement 740704.

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