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LETTER

Evolution of suicide as a defence strategy against pathogens in a spatially structured environment

Thomas W. Berngruber,[1,*] Sébastien Lion[1] and Sylvain Gandon[1]

[1]*Centre d'Ecologie Fonctionnelle et Evolutive (CEFE), UMR 5175, CNRS, 1919 route de Mende, 34293, Montpellier, France*

**Correspondence: E-mail: berngruber@gmail.com*

Abstract

Suicide upon infection by lytic phages is known in several bacteria species and represents an effective defence strategy to limit phage spread. However, the ecological conditions favouring the evolution of such a radically altruistic behaviour are unclear. Here, we model the feedback of epidemiology on host evolution in a spatially structured environment and we generate several specific predictions on altruistic suicide evolution. We test these predictions experimentally by competing *E. coli* cells carrying the suicide gene *Lit* against non-carrier cells in the presence or in the absence of the lytic phage T6. We show that in accord with our theoretical analysis altruistic suicide is only favoured in the presence of the phage in spatially structured environments at intermediate levels of mixing. Our work provides a general explanation for the evolution of altruistic defence strategies against pathogens. We discuss the implications of these results for oncolytic virus therapy.

Keywords

Cooperation, epidemic feedback, microbial ecology, pathogen-host interactions, spatial structure.

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INTRODUCTION

Programmed cell death is crucial in the development and the maintenance of multicellular organisms (Kerr *et al.* 1972). For instance, apoptosis is an important mechanism against the invasion of malignant cells, but it is also an efficient defence strategy against pathogens (Greenberg & Yao 2004; Coll *et al.* 2011; Fuchs & Steller 2011). The evolution of programmed cell death makes perfect sense at the scale of the organism where all cells share the same genes (Vaux *et al.* 1994; Gardner & Grafen 2009). In this case the cost of losing some cells is compensated by the benefit associated with preventing the spread of the infection to the whole organism. A similar reasoning may also explain the evolution of altruistic defence strategies in some social insect species (Shorter & Rueppell 2012). Here, again, the high relatedness among the individuals of the colonies may explain the suicidal behaviours that may lead infected individuals to leave the nest (Heinze & Walter 2010; Rueppell *et al.* 2010).

Numerous apoptosis mechanisms have been described in prokaryotic and eukaryotic single celled organisms (Engelberg-Kulka *et al.* 1998; Gardner & Kümmerli 2008; Nedelcu *et al.* 2011; Reece *et al.* 2011). In particular, some bacteria have been found to commit suicide just after being infected by a lytic virus (Shub 1994; Snyder 1995; Chopin *et al.* 2005). Altruistic suicide upon infection benefits the whole host population because it prevents the spread of the pathogen. But the evolution of self-sacrifice is problematic as the individuals who express this trait cannot directly transmit their genes (Vaux *et al.* 1994; Nedelcu *et al.* 2011). Kin selection theory provides a good framework to study the evolution of altruistic traits in microorganisms (West *et al.* 2006), but the feedback of epidemiology on host evolution requires specific theoretical developments. Altruistic suicide does not contribute to a common good, but, instead, delays a common threat (the infection) and acts as a firebreak against the spread of the epidemic (Fig. 1). It is necessary to take into account the spatial epidemiology of the pathogen to understand host evolution. Débarre *et al.* (2012) incorporated such epidemiological feedback into a general framework for the evolution of resistance and predicts that suicide upon infection can indeed evolve, but only if the pathogen is lethal and when infection occurs in a spatially structured environment. This prediction has recently been confirmed in spatially explicit simulations and in experiments that use an artificially engineered suicide mechanism (Fukuyo *et al.* 2012). To engineer such a suicide mechanism Fukuyo *et al.* (2012)

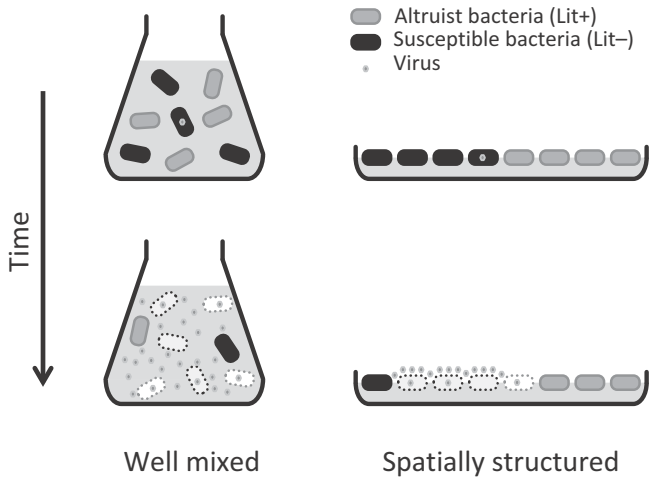

Figure 1 Altruistic suicide firebreak. In a well-mixed environment, suicide of infected hosts reduces virus density which benefits altruists and non-altruists, but only the altruist pays the cost of suicide and loses competition (eqn 2). In contrast, in a spatially structured environment, altruist hosts cluster and self-sacrifice of an altruistic cell spares other altruists from infection. This firebreak effect creates an extra benefit of suicide upon infection (eqn 3) which depends on the spatial epidemiology and spatial genetic structure of the host population. Interestingly, this extra benefit is strongest at intermediate levels of mixing (see Fig. 2). Too little mixing and the epidemic cannot take off. Too much mixing and spatial clustering is too low to build an epidemiological firebreak

expressed a methylation-specific restriction enzyme in *E. coli* and integrated its compatible methylase into the genome of phage λ. Phage infection thus triggers degradation of the bacterial genome and cell death. By this engineered suicide system they demonstrate that suicide has a selective benefit in a spatially structured environment (soft agar) but not in a well-mixed culture (liquid).

In the present article we further explore the importance of spatial structure on the evolution of altruistic suicide as a defence strategy against infections. Following and expanding the analysis of Débarre *et al.* (2012), we study the evolution of host defence strategy in the specific context of the interaction between bacterial hosts and their lytic phages. This analysis yields several new predictions in well-mixed, as well as in spatially structured environments. To test these predictions we investigated the selective advantage of the altruistic suicide gene *Lit* found in many genomes of *E.coli* K12 strains where it is part of the defective prophage e14 (Kao & Snyder 1988; Linder *et al.* 1994). In the absence of phage infection the Lit protein is expressed and accumulates in the host cell in an inactive form. During infection by a T-even phage (T2, T4 or T6) *Lit* changes its fold into an active form upon interaction with the phage major head protein. This active form of *Lit* acts as a protease that cleaves the ribosomal translation elongation factor Tu (EF-Tu) (Yu & Snyder 1994; Georgiou *et al.* 1998). Cleavage of EF-Tu leads to an immediate arrest of translation and ultimately to cell death before the infecting phage can complete its replicative cycle. Hence, the name of the gene *Lit* which stands for Late inhibition of T4. To test our theoretical predictions, we expressed a *Lit*-GFP fusion that fluorescently marks altruistic cells. Fluorescent tagging allowed us to monitor by flow cytometry the fate of altruistic (*Lit*) bacteria in competition with susceptible (*ΔLit*) bacteria in the presence (or absence) of T6. We manipulated the degree of mixing in spatially structured environments, followed the spatial distribution of altruistic (*Lit*) bacteria and studied the effect of mixing on the evolution of the bacteria. We show that, as expected from our theoretical analysis, intermediate levels of mixing are most favourable for the evolution of altruistic suicide because it allows efficient epidemic spread, but maintains sufficient spatial clustering of altruists (Fig. 1,2).

MATERIALS AND METHODS

Theoretical analysis

We developed a mathematical model to describe the competition between fully susceptible bacteria, *S*, and two defence strategies against pathogens (altruistic suicide, *A*, and classical resistance, *R*).The well-mixed model is described in the main text. The derivation of the spatially structured model is described in the supporting information.

Bacterial strains, plasmids, phage and media

We used a *Lit* genomic knock-out host (*Lit*::Kan Keio collection strain JW1125, NBRP National Institute of Genetics, Japan) carrying a plasmid that expresses a *Lit*-GFP fusion (JW1125 Aska + collection, NBRP National Institute of Genetics, Japan), hereafter referred to a p*Lit*-GFP. As a negative control we deleted a 553 nucleotide fragment from *Lit* (*Lit* position 66–619) by amplifying p*Lit*-GFP with primers CTCCTTCTCTGGTGCAATCT and GGCATTGCTACGGCAGTGCT and subsequent self ligation. This deletion in the *Lit* gene shifted the reading frame for the fused GFP and therefore annihilated green fluorescence. We will refer to this negative control as pΔ*Lit*-ΔGFP hereafter. For all infection assays we used phage T6 (NBRP National Institute of Genetics, Japan). All experiments were carried out in Luria Broth with 5 μM chloramphenicol (CM) to maintain plasmids, and 5 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) to express GFP.

One-step growth curves of T6

Cells carrying p*Lit*-GFP and pΔ*Lit*-ΔGFP were grown in exponential phase for 3 h, infected with T6 at a multiplicity of infection (MOI) of 0.01 and washed onto a 13 mm diameter, 0.2 μm pore-size polyvinylidene fluoride (PVDF) filter (Durapore, Milipore). Subsequently, the filter was rinsed to wash out unabsorbed phage. Thereafter, the filter was rinsed at 0.5 mL min^{-1} to elute newly produced phage and the effluent was collected at 5 min intervals. The pfu mL^{-1} titer of the collected effluent fractions was determined by top-agar plating and plaque counting on strain JW1125 carrying pΔ*Lit*-ΔGFP.

Bacterial growth curves

Cells carrying p*Lit*-GFP and pΔ*Lit*-ΔGFP were grown in exponential phase for 3 h, diluted to $OD_{600\ nm} = 0.1$ and subsequent growth was followed in 5 min intervals for uninfected cells, or cells infected with T6 at MOI = 0.01 and MOI = 10 in a micro-well plate reader (Infinite 200, Tecan, Austria). Each growth curve was measured in eight replicates. We verified that the drop in $OD_{600\ nm}$ corresponds to a drop in the number of living cells by counting colony forming units (CFU) at the end point of each growth curve.

Fitness cost of *Lit* and GFP

The cost of *Lit* in liquid was determined by mixing cells carrying p*Lit*-GFP and pΔ*Lit*-ΔGFP at a 1 : 1 starting frequency and a starting density of $OD_{600\ nm} = 0.09$ and growing them for 6 h at 37 °C in the absence or presence (MOI = 10^{-2}) of phage in 12 experimental replicates each. The cost of GFP in liquid was determined by competition of p*Lit*-GFP (JW1125 Aska +) against cells carrying the homologous plasmid without the GFP fusion in 12 experimental replicates (JW1125 Aska-). Cost of *Lit* and GFP in space was determined in the No-mixing environment (see next section) in three and six experimental replicates respectively. The starting frequency f_{start} and end frequency f_{end} of *Lit*-GFP were determined by flow cytometry (see below). The relative fitness *W* was calculated as:

$$W = (f_{end}/(1 - f_{end}))/(f_{start}/(1 - f_{start})).$$

Competition in space

We plated a 10^8fold dilution of a 1 : 1 mixture of cultures of p*Lit*-GFP and pΔ*Lit*-ΔGFP on 1% agar plates (5 μM CM and 5 mM IPTG) and grew overnight until we observed spatially structuring of densely packed colonies. This population structure was propagated onto fresh plates by a sterile velvet cloth. Thereafter, T6 was inoculated locally by dipping an array of 0.4 mm diameter stainless steel tattooing needles into a T6 lysate of 10^5 pfu mL^{-1} and poking it into the agar plate. By this procedure local T6 spots were inoculated

which are approximately 3 mm spaced apart. After inoculation of T6, spatial structure was either left undisturbed (No mixing) or mixed by agitation (600 rpm) with 4 mm diameter sterile stainless steel beads for 30 s once or during the entire time of incubation (24 h). In an additional 24 h treatment, we eliminated the microscopic spatial structure by overlaying the agar plate by a 3 mm layer of saline solution during agitation with steel beads (24 h-wet). We performed three experimental replications for each environment in the absence and presence of phage. Pictures of spatial structure were taken on an Olympus BH-2 RCF fluorescence microscope with a GFP-mCherry filter (Chroma #59022) and a custom 460(30) BP pre-filter (Chroma) using a 2× fluorite objective.

Quantification of GFP and ΔGFP cells by flow cytometry

Cells from the liquid competition were diluted 50 fold in saline solution and cells from the spatial competition were resuspended from the agar plate by an overlay with 1 mL saline solution and agitation at 600 rpm for 5 min. All flow cytometry was carried out on a FacsCalibure (BD Sciences, Franklin Lakes, NJ, USA) at 488 nm excitation and detection in the FL-1.

Statistical analysis

Analyses were carried out using the R statistical package (version 2.12.0). The general procedure was as follows. Models were built by including the presence (or absence) of the phage and the different mixing treatments (No mixing, 30 s, 24 h, 24 h wet) as fixed explanatory variables. Maximal models, including all higher-order interactions, were simplified by sequentially eliminating non-significant terms and interactions to establish a minimal model (Crawley 2007). A posteriori contrasts were carried out by aggregating factor levels together and by testing the fit of the simplified model using a likelihood ratio test (Crawley 2007).

RESULTS

Theory

To understand the evolution of altruistic suicide, we derive below a model to describe the epidemiology and the evolution of host defence strategies of *E. coli* bacteria against lytic phages. In the absence of infection, the bacteria are assumed to reproduce at a rate r and to die at a rate d. The parameter κ measures the intensity of density dependence which is assumed to decrease the fecundity of the bacterial population. We consider three types of hosts: fully susceptible bacteria (with density S), altruistic suicide bacteria (A) and classically resistant bacteria (R). The total density of the bacteria is $N = S + R + A$. We assume that each defence strategy may carry a cost, c_A and c_R, respectively, decreasing the growth rate of bacteria. For the sake of simplicity, we first describe the epidemiology and evolution of this system in a well-mixed model before discussing the effect of spatial structure.

Well-mixed model

We assume that, upon lysis, a susceptible bacterium releases a constant number B (burst size) of virus particles. Free virus may die at rate d_V or adsorb to both infected and uninfected bacteria at rate a. The adsorbed virus may enter the cell with a probability b. The above life cycle yields the following system of ordinary differential equations:

$$\begin{aligned}\frac{dN}{dt} &= (r(1 - c_A f_A - c_R f_R)(1 - \kappa N) - d - abV(1 - f_R))N \\ \frac{dV}{dt} &= (ab(1 - f_A - f_R)NB - d_V - aN)V\end{aligned} \quad (1)$$

Where $f_A = A/N$ and $f_R = R/N$ refer to the frequency of altruism and the frequency of resistance in the bacterial population respectively. The above equations show that host defence has a threefold effect on the epidemiological dynamics. First, if host defence is costly, a higher frequency of A and R bacteria can affect the overall bacteria population through a reduced growth rate. Second, higher levels of classical resistance (i.e. higher values of f_R) decrease the effect of the virus on overall population growth because resistant bacteria, in contrast with altruistic bacteria, are not killed upon infection. Third, host defences (i.e. both altruistic suicide and classical resistance) reduce the reproduction of the virus and this reduction in viral density can feed back on the growth of the bacteria population. In fact there is a threshold frequency of the total level of host defence, $f_A + f_R$, above which the virus cannot spread in the population: $f_c = 1 - 1/R_0$, where the basic reproduction ratio of the virus is $R_0 = abNB/(d_V + aN)$.

Interestingly, however, the frequencies of these two defence strategies are dynamical variables with the following dynamics:

$$\begin{aligned}\frac{df_A}{dt} &= -f_A(1 - f_A)rc_A(1 - \kappa N) + f_A f_R(rc_R(1 - \kappa N) - abV) \\ \frac{df_R}{dt} &= f_R(1 - f_R)(abV - rc_R(1 - \kappa N)) + f_R f_A rc_A f_A(1 - \kappa N)\end{aligned} \quad (2)$$

The above equations show that host defence evolution is governed by the balance between the cost and the benefit associated with each defence mechanism (see supporting information). For classical resistance, individuals paying the cost of resistance can increase in frequency when the force of infection is sufficiently high to compensate the cost of resistance (i.e. $abV > rc_R(1 - \kappa N)$). In contrast, altruistic suicide can never increase in frequency. At best, when there is no intrinsic cost of being an altruist, the evolution of altruistic suicide is neutral. In other words, in spite of the beneficial consequences of having a large number of altruists in the population (it limits the spread of the infection), altruistic suicide is not expected to evolve in a well-mixed population. This is because all the bacteria (be they altruistic or not) have the same probability of being killed by the infection. The evolution of altruistic suicide only makes sense if the benefit of this sacrifice is transferred preferentially to individuals carrying the same trait. Spatial structure and limited dispersal generate clusters of related individuals in which the cost of self-sacrifice to the individual may be outweighed by the benefit to its relatives (Débarre *et al.* 2012; Fukuyo *et al.* 2012) (Fig. 2).

Spatially structured model

Let us now assume that bacteria interact on a two-dimensional lattice. Each site of the lattice can either be empty ($\emptyset$), occupied by a susceptible bacteria (S), an altruistic bacteria (A) or by a resistant bacteria (R). The bacteria-phage interaction follows the same epidemiological dynamics as in the non-spatial model, except that reproduction and infection are now local processes (see supporting information). First, we assume that bacteria can only reproduce into empty neighbouring sites, which entails that density dependence is

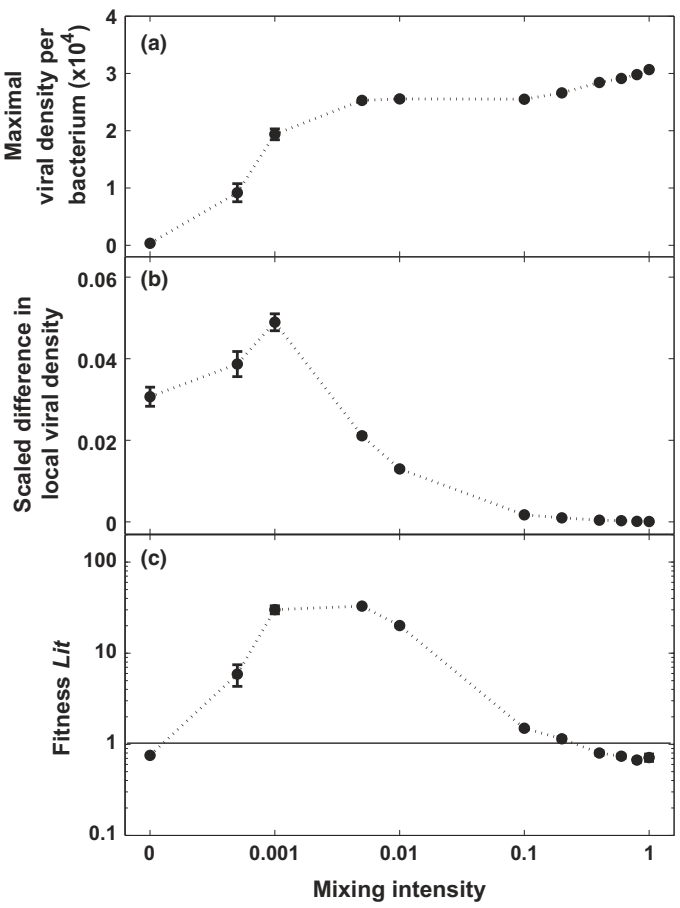

Figure 2 Simulation results on the evolution of altruistic suicide in a spatially structured environment. Effects of different levels of mixing ($m_B = m_V$) on (a) the maximum of the total density of the virus V, (b) the difference between the density of virus experienced by altruistic and susceptible hosts $\frac{v_S - v_A}{\bar{v}}$ (see eqn 3 in the main text), (c) the fitness of the altruistic suicide (*Lit*) gene. See supporting information for more details about the simulations and the parameter values (Dots: mean over 40 simulations and 95% CI)

local. Second, infection depends on the local density of phages experienced by bacteria. In a well-mixed population, both susceptible and altruistic bacteria will experience the same density of phages, but in a spatially structured population, the average density of bacteria experienced by susceptible and altruistic bacteria (noted v_S and v_A respectively) may differ. This is because local reproduction leads to the clustering of bacteria types, and the total production of phages will differ between clusters of susceptible and altruistic bacteria. The distributions of bacteria and viruses across space are shaped by the mixing rates, m_B and m_V respectively. We detail the analysis of this spatial model in the supporting information and we derive the change in frequency of both types of defence strategies. In the following, however, we focus on the evolution of altruistic suicide in the absence of classical resistance (i.e. $f_R = 0$):

$$\frac{df_A}{dt} = f_A(1-f_A)\left[\underbrace{-rc_A q_{o/A}}_{\text{direct cost}} + \underbrace{r\left(q_{o/A} - q_{o/S}\right)}_{\text{demography}} + \underbrace{ab\bar{v}\left(\frac{v_S - v_A}{\bar{v}}\right)}_{\text{epidemiology}}\right] \quad (3)$$

Where $q_{o/A}$ (resp. $q_{o/S}$) is the average local density of empty sites in the neighbourhood of a A (resp. S) bacteria, and $\bar{v}$ is the density of virus experienced by a bacteria. The first term in eqn 3 is the spatial analogue of the direct cost of altruistic suicide that we identified in the non-spatial model. However, eqn 3 reveals that additional forces are acting on the evolution of altruistic suicide in a spatially structured population.

First, altruistic suicide may be favoured if altruistic bacteria have a higher access to empty sites than susceptible bacteria ($q_{o/A} - q_{o/S} > 0$). Second, altruistic suicide may also get a selective advantage if, on average, altruistic bacteria are less exposed to lytic virus particles than susceptible bacteria ($\frac{v_S - v_A}{\bar{v}} > 0$). Note that, in the limit where the mixing rate of the population is high, one expects $q_{o/A} = q_{o/S}$ and $v_S = v_A$, so that the last two terms of the RHS of eqn 3 vanish and we recover eqn 2. Unfortunately, the complexity of the dynamics of the quantities $q_{i/j}$ and v_i hampers further analytical exploration of the evolutionary dynamics of altruism.

Equation 3, however, is very useful for understanding the stochastic simulations of this system. Fig. 2 presents a situation where bacteria and viruses have the same mixing rates (i.e. $m_B = m_V$). In contrast to the well-mixed model, our spatial stochastic simulations show that altruistic suicide can win in space, but that selection for altruistic suicide is maximised at intermediate levels of mixing. This is due to a twofold effect of mixing, which can be understood from the last term in eqn 3. First, mixing has a straightforward epidemiological effect and allows the epidemic to spread across the whole lattice. Second, mixing destroys the spatial distribution of viruses and hosts and therefore erodes the benefit of suicide upon infection: if altruistic bacteria tend to have relatively fewer altruistic neighbours, they will be exposed to more viral particles (see Fig. 1). The action of these two forces can be readily seen from the last term in eqn 3 which depends on both the average density of viruses $\bar{v}$ and the difference in the densities of viruses experienced by susceptible vs. altruistic bacteria $\frac{v_S - v_A}{\bar{v}}$. The first factor increases monotonically with the level of mixing, while the second decreases with mixing (Fig. 2). The combination of these two effects explains the non-monotonic relationship between the fitness of altruistic suicide and mixing. Additional simulations allow us to decouple the effects of bacteria and virus mixing rates (Fig. S1). An increase of bacteria mixing rate results in lower selection for the A strain because mixing reduces the genetic structure of the host population. In contrast, an increase in virus mixing rate has a non-monotonous effect. No virus mixing prevents the spread of the epidemic, but too much virus mixing removes the difference in the rate of exposition of the two types of hosts. This indicates that the non-monotonous effect observed when bacteria and virus mixing rates are constrained to be equal (Fig. 2) is due to the effect of virus mixing.

EXPERIMENTS

Lit reduces phage production

In the presence of T6 phage, the expression of *Lit* drastically reduces phage production after 70 min (t = 28.4235, d.f. = 3, $P = 10^{-4}$) (Fig. 3). To study how this reduction in phage production affects bacterial growth in the presence of T6, we infected *Lit* and *ΔLit* cultures at a low MOI. For an initial MOI = 0.01 we observe that a population of bacteria that expresses a functional *Lit* can grow in the presence of T6 whereas a *ΔLit* population phage rapidly collapses due to the multiplication and spread of T6 (Fig. 4b).

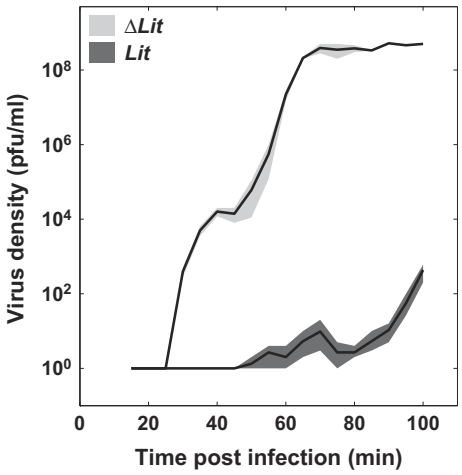

Figure 3 One-step growth curve of T6 on susceptible *ΔLit* and altruistic *Lit* carrying bacteria. A T6 infected *ΔLit* culture produces two consecutive bursts within 70 min (first burst 40 min) and reaches total titre of 5.10^8 pfu mL^{-1} (mean and min-max bounds for the light gray shaded area, two replicates). In the same time-span (70 min), a T6 infected *Lit* culture only produces a single burst of about 20 pfu mL^{-1} (mean and min-max bounds for the dark gray shaded area, three replicates) and the overall production of phage is drastically reduced. All cultures were initially infected at MOI = 0.01

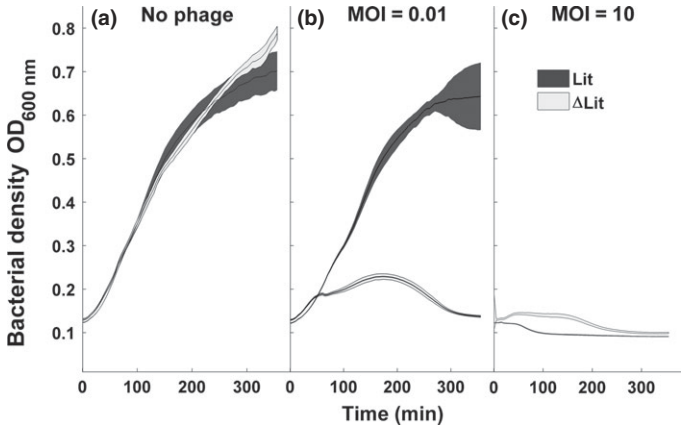

Figure 4 Growth and epidemic dynamics of susceptible (*ΔLit*) bacteria and altruistic (*Lit*) bacteria. (a) Increase in optical density ($OD_{600\ nm}$) of a pure *ΔLit* culture and a pure *Lit* culture in the absence of phage. (b) Epidemic collapse of a pure *ΔLit* culture and a pure culture of *Lit* cells infected at MOI = 0.01. Note that in the *Lit* culture epidemic collapse is delayed. (c) Epidemic collapse of a pure *ΔLit* culture and a pure culture of *Lit* cells infected at MOI = 10. Note that altruistic suicide causes collapse of the *Lit* culture even before epidemic collapse of the *ΔLit* culture (Solid line: mean $OD_{600\ nm}$ for eight independent replicate cultures. Shaded area: 95% CI)

Is *Lit* truly acting by cell suicide?

To determine whether *Lit* reduces viral production truly by altruistic suicide or by a mechanism that provides classical resistance (without suicide) we infected cells at high multiplicity of infection. At MOI = 10 all cells are infected and we therefore expect that *Lit* expressing cells should all be killed by cell suicide immediately after infection. This effect is clearly demonstrated in (Fig. 4c). Interestingly, upon infection, the OD of the *Lit* cultures drops earlier than that of the *ΔLit* cultures. This means, that altruistic suicide kills the cells even before the virus completes its lytic cycle and confirms that *Lit* cells truly commit suicide upon infection by T6.

Lit reduces bacterial growth rate in the absence of T6

In the absence of phage T6, the expression of the p*Lit*-GFP fusion is costly. To control for the cost of GFP we competed p*Lit*-GFP against a homologous plasmid without GFP (see methods) and found no significant cost of GFP ($t = 0.49$, d.f. = 16, $P = 0.63$) and no effect of the environment ($F_{1,16} = 0.29$, $P = 0.6$). In contrast, the expression of *Lit* causes a significant fitness cost ($t = 21.8$, d.f. = 13, $P < 10^{-4}$) in both environments ($F_{1,13} = 0.15$, $P = 0.7$) (Fig. 5). This demonstrates that *Lit* has a toxic effect on the cells, which might either originate from the expression cost of the Lit protein (reduced fecundity) and/or from erroneous stochastic triggering of the suicide mechanisms (increased mortality).

Can *Lit* increase in frequency in competition with Δ*Lit* in a liquid environment?

We monitored the competition between *Lit* and *ΔLit* bacteria in liquid cultures for 6 h. In the liquid environment *Lit* also has a significant fitness cost in the presence of phage T6 ($t = 78.3401$, d.f. = 11, $P < 10^{-4}$, data not shown). Hence, in liquid, *ΔLit* cells lose competition against *Lit* cells, both, in the presence and absence of T6. We can therefore conclude that, as predicted in eqn 2, the altruistic suicide gene *Lit* is counter selected in a liquid environment.

Can *Lit* increase in frequency in a spatially structured environment?

To investigate whether the altruistic suicide mechanism of *Lit* can increase in frequency in a spatially structured environment, we monitored the competition between *Lit* and *ΔLit* cells on agar plates in a fully structured environment (No mixing treatment). We also manipulated the degree of mixing by placing 4 mm diameter sterile

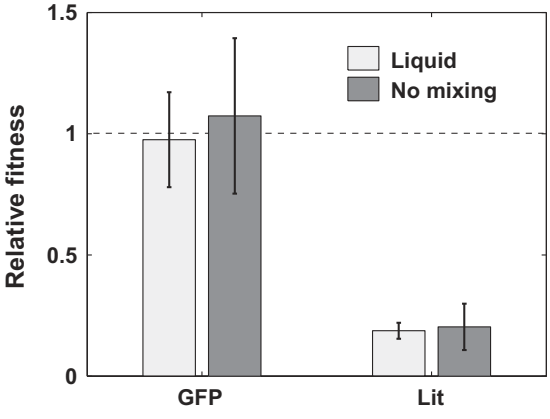

Figure 5 Fitness costs of GFP and *Lit* in the absence of phage T6. Expression of GFP has no effect on fitness in both liquid and spatially structured environments. The expression of *Lit*, however, does cause a significant fitness cost both in liquid and in a spatially structured environment. Bars refer to the mean fitness ± 95% CI

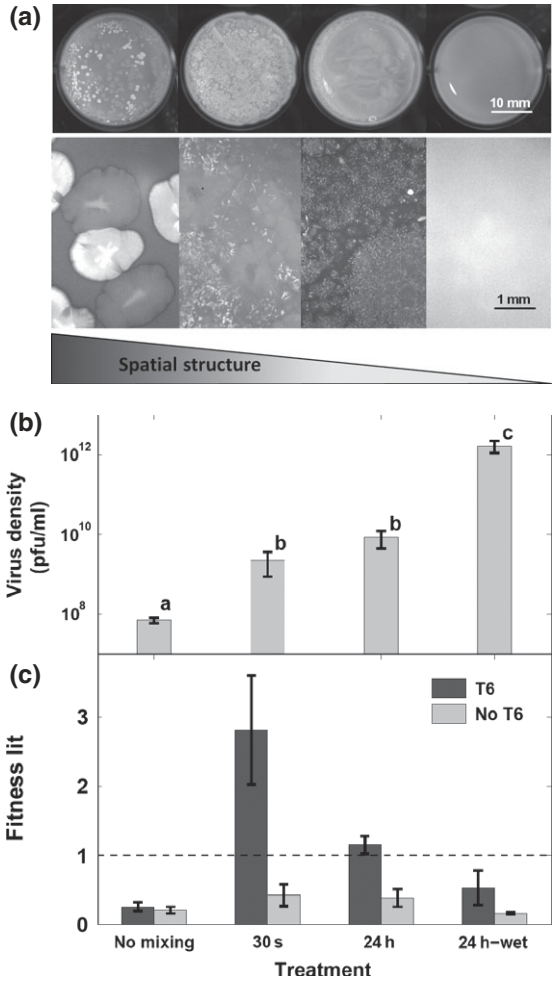

Figure 6 Spatial structure affects the relative fitness of the altruistic suicide gene *Lit*. (a) Spatial structuring of *Lit* and *ΔLit* micro colonies. Fluorescent (bright) colonies: *Lit-GFP;* dark colonies: non-fluorescent *ΔLit-ΔGFP*. Top - macroscopic structure. Bottom - 20× magnification. Left to right - increasing degrees of mixing (No mixing, 30 s, 24 h, 24 h-wet). (b) Final phage titre of the T6 epidemic. (c) Fitness of *Lit* bacteria for different mixing treatments (Bars: means of three replicates ± SE). Note that the fitness of *Lit* is always below one in the absence of phage T6, but above one at intermediate mixing treatments which allow efficient epidemic spread while maintaining clustering of altruists [see treatment 30 s in (a) and (b)]

stainless steel beads on Petri dishes and agitating for variable amounts of time (30 s, 24 h or 24 h-wet, see Materials and Methods). This disturbance of spatial structure has a direct effect on the scale of clustering of altruistic *Lit* carriers (Fig. 6a). Furthermore, mixing increases long-range transmission of phage T6 and leads to a strong increase of the production of viral particles ($F_{3,8} = 8.92$ $P = 0.006$) (Fig. 6b). We found that mixing had no effect on the competitive ability of *Lit* in the absence of phage T6 ($F_{3,8} = 1.53$ $P = 0.28$) (Fig. 6c). In the absence of T6, the mean competitive ability of *Lit* is below one ($W_{Lit} = 0.29 \pm 0.12$, mean ± 95%CI) and *Lit* loses competition against *ΔLit* in all treatments. Yet, in the presence of phage T6 mixing has a strong effect on the competitive ability of *Lit* ($F_{3,8} = 7.53$ $P = 0.01$). Whereas *Lit* loses competition in the fully structured environment (No mixing) and in the fully mixed environment (24 h-wet), it can increase in frequency with intermediate mixing (30 s, Fitness $W_{Lit\text{-}30s} = 2.81 \pm 1.88$, mean ± 95%CI) (Fig. 6c).

In summary, we found that the altruistic suicide gene *Lit* can only increase in frequency in a spatially structured environment at an intermediate level of mixing (see Fig. 6c). We explain this effect by the dual effect of spatial structure on epidemiology and relatedness. On the one hand, disturbance of spatial structure facilitates spread of the virus and leads to an overall increase in virus density (see Fig. 6b). On the other hand, spatial mixing disrupts the clustering of altruists and reduces the proportion of the benefit of altruistic suicide that is directed to other altruists (see Fig. 6a). The balance between these two opposing forces yields maximal selection on *Lit* for an intermediate level of mixing that enables efficient propagation of the epidemic and maintains sufficient clustering of altruist individuals (see Fig. 6c).

DISCUSSION

The evolution and maintenance of self-sacrifice in single-celled organisms is a theoretical challenge. Here, we show that suicide upon infection can evolve as an altruistic defence strategy against lytic phages in bacteria. As pointed out by kin selection theory, spatial structure plays a key role in this evolution (West *et al.* 2006). Environmental viscosity generates clusters of related bacteria which allow the benefits of self sacrifice (i.e. limited exposition to phages) to be directed preferentially towards related individuals (Débarre *et al.* 2012). Our theoretical analysis reveals, however, that spatial structure is not enough to select for altruistic suicide. This is because, unlike classical altruistic acts where the benefit to your neighbours is a fixed quantity (Hamilton 1964), the benefit generated by altruistic defence against pathogens is a dynamical variable. A full understanding of the evolution of this trait needs to take into account epidemiological dynamics. In particular, we show that altruistic defence strategies are only expected to evolve at intermediate levels of mixing. When mixing is too low, the virus cannot spread in the host population and there is no selection for resistance. When mixing is too high, the virus spreads easily but the absence of spatial structure prevents the evolution of altruistic suicide (see Fig. 2 and Fig. 6c).

We developed a new experimental model and a new experimental design to explore the effect of mixing on the evolution of altruistic suicide. First, we confirmed that *Lit* is a costly but efficient defence strategy acting by altruistic suicide Figs 3, 4 and 5. Second, we realised competition experiments in the presence or in the absence of phages to validate our theoretical predictions: intermediate levels of mixing maximise selection for altruistic resistance.

We did not find evidence of phage evolution in our experiments but phage mutations that prevent the triggering of host suicide are likely to appear later on. Indeed, such mutations have been well described in a 75 bp region of the phage major head protein which is recognised by *Lit* (Georgiou *et al.* 1998). Evolution may also take place in the host. Classical resistance against the pathogen may appear by mutation and challenge the altruistic suicide strategy. In *E. coli* the acquisition of classical resistance to T-even phages is well described and occurs at a mutation rate of approximately 10^{-8} per generation (Demerec & Fano 1945). Our theoretical model indicates that at such low mutation rates, the occurrence of classical resistance does not alter qualitatively our short-term predictions (Fig. S2). In the long-term, however, classical resistance may take over.

The potential evolution of escape mutations in the virus and classical resistance in the host raises the question of the long-term maintenance of altruistic suicide in bacterial populations. Three factors may play a key role in the maintenance of altruistic suicide. First, altruistic suicide is an inducible defence mechanism that is triggered only in the presence of phage. In the absence of phage the expression of the suicide machinery might be less costly than classical resistance (i.e. $c_A < c_R$). An evaluation of the fitness costs of altruistic and more classical defence strategies is difficult in our system because the cost of the altruistic suicide machinery is likely to be inflated by the over-expression of *Lit* from a plasmid. The comparison between the direct costs associated with these different defence strategies remains to be carried out in *E. coli* and in other bacteria. Second, a single altruistic suicide mechanism can be active against several different phages (Snyder 1995; Engelberg-Kulka *et al.* 1998; Chopin *et al.* 2005). Classical resistance, in contrast, is often very specific (e.g. Henning & Jann 1979) and its direct cost can be considerable (Lenski 1988). The diversity of the phage community may thus explain the maintenance of more generalist defence strategies like altruistic suicide. Third, the maintenance of altruistic defence strategies could certainly be facilitated by horizontal transfer between bacteria. Noteworthy, *Lit* gene is part of the deficient prophage e14 which is no longer able to actively replicate and transmit horizontally. This suggests that, while it was still replicating the phophage e14 might have facilitated the invasion of *Lit* into the *E.coli* population. In fact, the ability for horizontal spread seems to be common in prokaryotic suicide systems, which are often coded by mobile elements like prophages, transposons and plasmids (Snyder 1995; Pecota & Wood 1996; Chopin *et al.* 2005). Well known examples include the *rex* gene products of phage λ, the *pif* gene product of the F plasmid and the *prrC* gene which resides on a cryptic DNA element related to phage P1 in some clinical isolates of *E. coli* (Snyder 1995; Georgiou *et al.* 1998). Such horizontal transmission of altruistic genes has been predicted to facilitate the evolution of altruistic traits, as non-altruist cheaters could be reinfected with the altruist gene (Smith 2001). Yet, the role of horizontal transfer of altruistic suicide mechanisms for the evolution of altruistic suicide as a defence against infection is an important open question for theoretical and empirical research.

Understanding how and why parasites use apoptosis could provide some ways to manipulate the cues that trigger the death of pathogens using their own cellular machinery. Harnessing the programmed cell death of microbes could thus provide a novel approach to fight many infectious diseases (Reece *et al.* 2011). The apoptosis induced by viral infection may also have important applications in the field of oncolytic viral therapy. Here, the idea is to use virus to control and ultimately, eradicate the spread of cancer cells in a patient (McCormick 2001; Chiocca 2002). Different strategies are being explored but one of the first study was based on the use of an adenovirus mutant ONYX-015 that lacks the ability to block p53 function of the host cell (Bischoff *et al.* 1996). ONYX-015 can infect both healthy and cancerous cells but cannot finalise replication in healthy cells which trigger altruistic suicide upon infection (McCormick 2001; Chiocca 2002). This should promote the local accumulation of viral particles around tumour tissue, and may thus enhance the efficacy of the treatment. This feedback of viral dynamics on the dynamics of malignant cells is analogous to the effect of spatial structure we report in the present study. In a spatially structured environment, the phage T6 accumulates around the *ΔLit* cells (the 'cancer' cells that do not perform apoptosis) and thus favour the growth of the *Lit* cells (the 'healthy' cells that perform apoptosis). Both scenarios can potentially create positive selection for apoptosis and altruistic suicide. Our results demonstrate that a better understanding of the efficacy of replicating oncolytic virus requires an explicit description of the spatial spread of the virus in the patient (Wodarz *et al.* 2012).

Our work confirms that altruistic suicide may evolve in unicellular organisms as a successful defence strategy against lethal infections. As in classical kin selection models, some level of spatial structure is required for the evolution of altruistic suicide. Yet, too much spatial structure can prevent the spread of the virus and hampers selection for resistance. These antagonistic effects explain why altruistic suicide evolution is favoured for intermediate rates of mixing. Our experimental results with *E. coli* and T6 confirm our theoretical predictions. Combining mathematical modelling with experimental evolution is a powerful tool to better understand the origin and the maintenance of these peculiar defence strategies against pathogens.

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AUTHORSHIP

SG, SL and TB designed experiments, TB performed the experiments. SL, SG carried out mathematical analyses and simulations, TB, SL and SG wrote the paper.

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