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

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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; Nedelec 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; Nedelec 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. Déharce 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)

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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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 pro tease 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 as 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 CTCCCTTCTCTGGTGTGCAATCT and GGCATTGC-TAGGGCAGTGTCT 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-thiogalacto-pyranoside (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 phu 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 \(f_{\text{start}}\) and end frequency \(f_{\text{end}}\) of *Lit*-GFP were determined by flow cytometry (see below). The relative fitness \(W^\prime\) was calculated as:

\[
W^\prime = \frac{f_{\text{end}}/(1-f_{\text{end}})}{f_{\text{start}}/(1-f_{\text{start}})}. \]

**Competition in space**

We plated a 10\(^{8}\) fold 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\(^{9}\) 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 FacsCalibur (BD Sciences, Franklin Lakes, NJ, USA) at 488 nm excitation and detection in the PI-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). Interestingly, however, the frequencies of these two defence strategies are dynamical variables with the following dynamics:

\[
\begin{align*}
\frac{df_A}{dt} &= -f_A (1-f_A) c_A (1 - \kappa N) + f_A (f_R (1 - \kappa N) - ab V) \\
\frac{df_R}{dt} &= f_R (1 - f_R) (ab V - r_A (1 - \kappa N)) + f_R (1 - f_R) c_R f_A (1 - \kappa N)
\end{align*}
\]

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. \(ab V > r_A (1 - \kappa N)\)). 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 (Debarre 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 (0), occupied by a susceptible bacteria (S), an altruistic suicide (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
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 $v_\text{S}$ and $v_\text{A}$ 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, $m_\text{B}$ and $m_\text{V}$ 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. $f_R = 0$):

$$
\frac{df_\text{A}}{dt} = f_\text{A}(1 - f_\text{A}) \left[-r_A q_{\text{A/A}} + r (q_{\text{A/A}} - q_{\text{S/S}}) + ab\left(\frac{v_\text{A} - v_\text{S}}{\bar{v}}\right)\right]
$$

(3)

Where $q_{\text{A/A}}$ (resp. $q_{\text{S/S}}$) 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 ($q_{\text{A/A}} - q_{\text{S/S}} > 0$). Second, altruistic suicide may also get a selective advantage if, on average, altruistic bacteria are less exposed to lytic virus particles than susceptible bacteria ($\bar{\rho}_\text{A} > \bar{\rho}_\text{S}$). Note that, in the limit where the mixing rate of the population is high, one expects $q_{\text{A/A}} = q_{\text{S/S}}$ and $v_\text{S} = v_\text{A}$, 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 $q_{\text{i/j}}$ 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. $m_\text{B} = m_\text{V}$). 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 $\bar{\rho}_\text{A} - \bar{\rho}_\text{S}$. 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, $P = 10^{-5}$) (Fig. 3). To study how this reduction in phage production affects bacterial growth in the presence of T6, we infected Lit and ALit 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 ALit population phage rapidly collapses due to the multiplication and spread of T6 (Fig. 4b).
that altruistic suicide causes collapse of the Lit culture and a pure culture of $A\text{Lit}$ culture even before epidemic collapse is delayed. (c) Epidemic collapse of a $A\text{Lit}$ 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 3 One-step growth curve of T6 on susceptible $A\text{Lit}$ and altruistic Lit carrying bacteria. A T6 infected $A\text{Lit}$ 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 ($A\text{Lit}$) bacteria and altruistic (Lit) bacteria. (a) Increase in optical density (OD$_{600 \text{ nm}}$) of a pure $A\text{Lit}$ culture and a pure Lit culture in the absence of phage. (b) Epidemic collapse of a pure $A\text{Lit}$ 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 $A\text{Lit}$ 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 $A\text{Lit}$ culture (Solid line: mean OD$_{600 \text{ nm}}$ 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 $A\text{Lit}$ 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 pLit-GFP fusion is costly. To control for the cost of GFP we competed pLit-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. $= 13$, $P < 10^{-5}$) 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 $A\text{Lit}$ in a liquid environment?**

We monitored the competition between Lit and $A\text{Lit}$ 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. $= 11$, $P < 10^{-5}$, data not shown). Hence, in liquid, $A\text{Lit}$ 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 $A\text{Lit}$ 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](image)

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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 ($\hat{D}_6 = 8.92$ $P = 0.006$) (Fig. 6b). We found that mixing had no effect on the competitive ability of Lit in the absence of phage T6 ($\hat{D}_6 = 1.53$ $P = 0.28$) (Fig. 6c). In the absence of T6, the mean competitive ability of Lit is below one ($\hat{W}_{Lit} = 0.29 \pm 0.12$, mean $\pm 95\%$CI) and Lit loses competition against $\Delta Lit$ in all treatments. Yet, in the presence of phage T6 mixing has a strong effect on the competitive ability of Lit ($\hat{D}_6 = 7.53$ $P = 0.01$). 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 $\hat{W}_{Lit,r30} = 2.81 \pm 1.88$, mean $\pm 95\%$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 exposure to phages) to be directed preferentially towards related individuals (Dèbarre 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. $c_A < c_B$). 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 $Llt$ 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 (Lensi 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, $Llt$ 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 prophage e14 might have facilitated the invasion of $Llt$ 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 $\lambda$, the $\psi$ 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 pathogen using their own cellular machinery. Harnessing the pro-

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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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