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LETTER

Multicellular organization in bacteria as a target for drug therapy

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Abstract

Antibiotic treatments are now reaching the limit of their efficiency, especially in hospitals where certain bacteria are resistant to all available drugs. The development of new drugs against which resistance would be slower to evolve is an important challenge. Recent advances have shown that a potential strategy is to target global properties of infections instead of harming each individual bacterium. Consider an analogy with multicellular organisms. In order to kill an animal two strategies are possible. One can kill each of its cells individually. This is what antibiotics do to get rid of bacterial infections. An alternate way, for instance, is to disorganize the hormonal system of animal's body, leading eventually to its death. This second strategy could also be employed against infections, in place of antibiotics. Bacteria are indeed often involved into coordinated activities within a group, and certain drugs are able to disorganize these activities by blocking bacterial communication. In other words, these drugs are able to target infections as a whole, rather than individuals within infections. The present paper aims at analysing the consequence of this peculiarity on the evolution of bacterial resistance. We use a mathematical model, based on branching process, to calculate the fixation probability of a mutant resistant to this type of drug, and finally to predict the speed of resistance evolution. We show that this evolution is several orders of magnitude slower than in the case of antibiotic resistance. The explanation is as follows. By targeting treatments against adaptive properties of groups instead of individuals, we shift one level up the relevant unit of organization generating resistance. Instead of facing billions of bacteria with a very rapid evolutionary rate, these alternate treatments face a reduced number of larger organisms with lower evolutionary potential. In conclusion, this result leads us to emphasize the strong potential of anti-bacterial treatments aiming at disorganizing social traits of microbes rather than at killing every individual.

Keywords

Anti-bacterial treatments, bacteria, cooperation, quorum-sensing, resistance.

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INTRODUCTION

Resistance to antibiotics is spreading among bacteria, compromising the efficiency of drugs (Heinemann 1999). Some genotypes are even resistant to all known medicine (Hiramatsu *et al.* 1997). To face this challenge, it is necessary both to develop new drugs and to evaluate the probability that bacteria could become resistant to them, in order to minimize the chance of treatment failure.

Among the new therapeutic strategies recently proposed, one of the most original and seducing is the attempt to disturb cooperation between clustered bacteria. Bacteria indeed are comparable with multicellular organisms or eusocial insects in many aspects of their lifestyle (Crespi 2001), i.e. individual cells are often involved into coordinated activities within a group. For instance, they communicate to control protein secretion (Williams *et al.* 2000; Brown & Johnstone 2001; Brown *et al.* 2002; West & Buckling 2003), they may differentiate and produce an extracellular matrix (Costerton 1999), or they can manipulate host's behaviour or physiology to the benefit of the entire bacterial infection (Brown 1999). These traits are

costly to express for each individual bacterium whereas they benefit to the group as a whole, hence they are said to be 'cooperative'. Precisely, the drugs developed so far to target cooperation interfere with a communication system of bacteria called quorum-sensing (Williams *et al.* 2000). Quorum-sensing is a two-component communication system. Each bacterium secretes a diffusible signal and expresses a corresponding receptor. When the density of bacteria is important, the concentration of signals measured by receptors exceeds a threshold, above which certain virulence factors are expressed and secreted outside of bacterial cells (e.g. siderophores, West & Buckling 2003; Griffin *et al.* 2004). These factors are cooperative because, being shared by a cluster of bacteria, their expression does not yield any particular advantage to the secreting individuals but only a global advantage to the cluster. Further, the blockade of quorum-sensing by analogues of communication molecules has been shown *in vitro* to inhibit efficiently the secretion of these factors (Eberhard *et al.* 1986; Passador *et al.* 1996; Schaefer *et al.* 1996; McClean *et al.* 1997; Swift *et al.* 1997, 1999; Balaban *et al.* 1998; Finch *et al.* 1998; Mayville *et al.* 1999; Alksne 2002).

Such treatments are interesting not only because they propose new targets to drugs, but also because the potential for the evolution of resistance to them is probably smaller than to conventional antibiotics. Let us indeed consider the case of a treatment preventing the secretion of a given virulence factor. Cooperation, i.e. the secretion of the virulence factor, is beneficial only at the higher level. Entire groups of bacteria that are all expressing the virulence factor are favoured over non-expressing groups. Prior to treatment, such cooperative trait was maintained by natural selection because groups are made of kin-related individuals, sharing cooperation genes through a recent common ancestor (Hamilton 1972). Specifically, virulence-expressing individuals were benefiting from the virulence factors produced by their related neighbours. Consider then a group of sensitive bacteria undergoing treatment. Each bacterium of the group is prevented from secreting the virulence factor, which is costly to the group as a whole. In other words, the treatment artificially turns cooperative bacteria into selfish individuals. Consider then a rare resistant mutant, able to express virulence despite the treatment. Initially this mutant does not share the cooperative gene (namely the resistance mutation) with its neighbours. Therefore, in the first stages of its existence, it is experiencing the strong cost of cooperation without receiving the beneficial counterparts from neighbours. Concretely in the case of virulence expression, the proteins secreted by the resistant mutant are shared among the entire group; hence, they do not benefit more to itself than to its sensitive neighbours. Therefore, the mutant is not favoured by local competition, and is even selected against because it produces expensive proteins to the benefit of the entire group (Maynard-Smith 1982; Brown *et al.* 2002; West & Buckling 2003). Indeed, observations and experimental competitions have shown that exo-proteins secretion is counter-selected in unstructured bacterial populations (Chao & Levin 1981; De Vos *et al.* 2001; Griffin *et al.* 2004).

In general terms, when bacteria are treated with an anti-cooperative drug, resistance to the drug is itself a cooperative trait and is therefore never favourable within groups. Such initial counter-selection against resistant mutants could have important consequences on the rapidity of resistance evolution. The aim of the present paper is to build a mathematical model to measure these consequences. Very generally, we consider any cooperative trait beneficial to entire bacterial groups. Depending on the trait considered, relevant groups can be merely few bacteria or entire infections; in all cases we refer to them as 'clusters'. We choose the simple, and yet conservative, situation where each cluster is made of a single bacterial clone; hence, the cooperative trait is very strongly favoured. A treatment is then applied that blocks cooperation (called anti-cooperative treatment), and a resistant mutant is considered. The probability that this mutant generates global resistance is calculated and then, considering the recurrent production of mutants, the likelihood of resistance evolution is estimated. This model will apply to any type of anti-cooperative treatments; nevertheless, the concrete application we have in mind is that of quorum-sensing blockade.

THE MODEL

We consider a large cluster-structured bacterial population treated with an anti-cooperative drug. In the present approach, we assume that the treatment is unable to lead to the ultimate extinction of all bacteria. After the treatment has started, the number of bacteria decreases from its natural level to a new equilibrium with n clusters containing each N bacteria. The pre-equilibrium phase, taking place just after the beginning of treatment, is neglected. Our aim is to calculate the rate of resistance evolution in the remaining treated population at equilibrium. Namely the probability that, at each unit of time, a resistant mutation appears and that this mutation reaches global fixation. In order to test the robustness of the results, in Appendix S3 (see Supplementary Material) we also consider an alternate model where the bacterial population does not reach equilibrium but is instead ultimately cleared by treatment. This model brought essentially the same results as the ones described here.

The number of resistant mutations produced per unit of time is unN, where u is the mutation rate of each bacterium towards resistance. The total rate of resistance evolution is then $R = unNP$, where P is the probability for a given resistant mutant to reach global fixation in the population

instead of being lost in the first stages of its existence. In the following we derive this probability P.

Resistant mutants appear in predominantly sensitive clusters, and need to reach fixation in a meta-population made of several clusters. The fixation process of a mutation thus has two distinct stages (see Fig. 1). First, the mutation fixes within a single cluster, which necessitates the dispersion of a mutant to an empty patch (Fig. 1); this occurs with a probability P_F. Second, the mutation fixes in the entire meta-population, after having reached fixation within a cluster; this occurs with a probability $P_{|F}$. The overall probability of fixation is the product $P = P_F \cdot P_{|F}$. In the following we derive P_F and $P_{|F}$.

Probability of resistance fixation within a cluster, P_F

The fate of resistant mutants is described as a continuous time branching process (e.g., see Iwasa *et al.* 2004 and Antia *et al.* 2003 for a discrete time equivalent). Consider a rare resistant mutant at time t within a focal cluster and define $K(t)$ as the probability that this mutant is ultimately lost. The resistant mutant is rare in the first place. It is expressing a cooperative trait in a predominantly selfish cluster. Therefore, the mutant does not have any significant advantage within its cluster and is even counter-selected. Mathematically, the single resistant mutant has replication and death rates r and μ, with $\mu > r$, i.e. the mutant produces less than one copy of itself in its entire life ($r/\mu < 1$). The mutant may also disperse from its cluster at a rate δ, in which case it has a probability s to survive in the external environment and establish its own cluster. On top of that, sensitive clusters undergo catastrophic extinctions at a rate d. This can be due to various types of events, such as host death or immune clearance, if the bacteria are part of an infection. Finally, $K(t)$ can be expressed by considering all the events occurring during an infinitesimal period dt:

$$\begin{aligned} K(t) = {} & r\mathrm{d}t(K(t+\mathrm{d}t))^2 \\ & + \mu\mathrm{d}t + d\mathrm{d}t + \delta\mathrm{d}t(1-s) \\ & + \delta\mathrm{d}t \times s \times K_{|F}(t+\mathrm{d}t) \\ & + (1 - r\mathrm{d}t - \mu\mathrm{d}t - d\mathrm{d}t - \delta\mathrm{d}t)K(t+\mathrm{d}t). \end{aligned} \quad (1)$$

In the first line, the bacterium divides (with a probability $r\mathrm{d}t$), if it is the case it will ultimately be lost if the two daughter cells are ultimately lost [probability $(K(t+\mathrm{d}t))^2$]. In the second line, the bacterium disappears because it dies (probability $\mu\mathrm{d}t$), because the cluster goes extinct as a whole (probability $d\mathrm{d}t$), or because it disperses (probability $\delta\mathrm{d}t$) and fails to found a new cluster (probability $1 - s$). In the third line, the bacterium disperses and succeeds in establishing a cluster, if it is the case it will ultimately be lost if the founded cluster is ultimately lost as a whole [probability $K_{|F}(t+\mathrm{d}t) = 1 - P_{|F}(t+\mathrm{d}t)$]. Finally, the bacterium can remain unchanged from t to $t+\mathrm{d}t$, in which case it is ultimately lost with a probability $K(t+\mathrm{d}t)$ (fourth line).

In order to simplify eqn 1, we make use of two useful properties of resistance. First, resistance is counter-selected within clusters ($r/\mu < 1$). As a result, a resistant mutation never reaches a significant frequency within the cluster where it first appeared. The cluster always remaining, as a result, largely dominated by sensitive bacteria, its ecological properties are constant through time. Namely, the extinction rate d and population size N of the cluster are fixed parameters, and so are the replication, mortality and dispersal rates of the rare mutants present inside the cluster (r, μ and δ). As a result, the probability for a given mutant

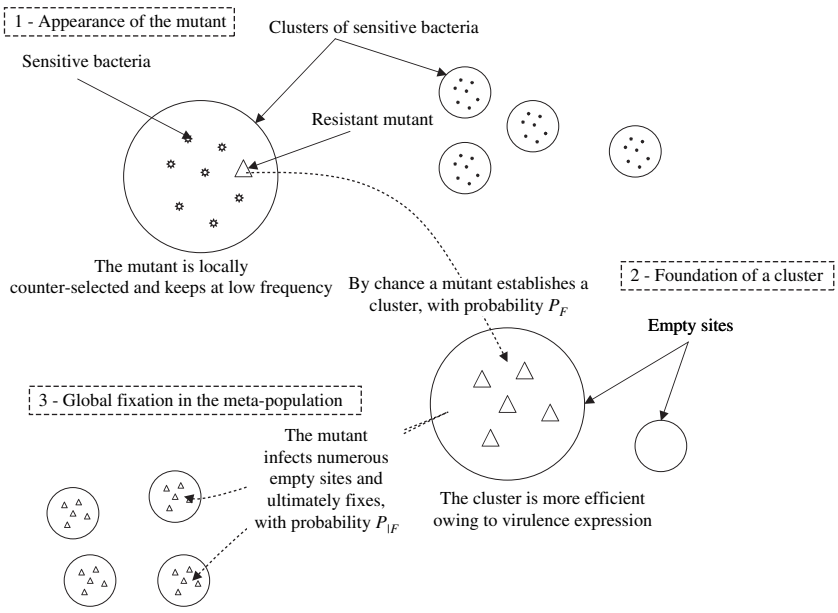

Figure 1 Schematic for the process leading to resistance fixation.

to be lost, $K(t)$, can be considered as independent of time ($dK/dt = 0$). Second, resistance is assumed to be rather strongly favoured at the level of clusters. Therefore, as soon as a small number of clusters are entirely resistant, then the emergence of resistance becomes a deterministic process. Fixation is uncertain only when the number of resistant clusters is still extremely low. In other words, during the whole stochastic process, the meta-population remains largely dominated by sensitive clusters and its ecological properties are constant through time (see Appendix S2). Therefore, the probability for a resistant cluster to be lost, $K_{|F}(t)$, can also be considered as independent of time ($K_{|F}(t) = K_{|F}$).

Equation 1 yields

$$rK^2 + \mu + d + \delta(1 - s) + \delta s K_{|F} - (r + \mu + d + \delta)K = 0,$$

which gives an analytical expression for $P = 1 - K$, the probability for a single mutant cell to generate eventually global resistance (see Appendix S1). In order to gain some intuitive insights from the analytical expression of the results, we consider the case where bacterial dispersal rate is weak, and express P with a Taylor development to the first order in δ. This gives

$$P = \frac{\delta s P_{|F}}{(\mu - r + d)} + o(\delta).$$

For details see Appendix S1.

Probability of resistance fixation after cluster foundation, $P_{|F}$

The probability of fixation after successful dispersal is calculated from a continuous time branching process, by considering the reproduction and extinction of resistant clusters. This derivation is detailed in Appendix S2 and yields

$$P_{|F} = 1 - \frac{S - n'}{S - n}, \quad (2)$$

where S is the total number of sites in the meta-population that are available for bacterial colonization, n' is the number of occupied sites (living clusters) when all bacteria are resistant to treatment, and we recall that n is the number of occupied sites when all bacteria are sensitive to treatment.

Rate of evolution

In the case of weak dispersal, the overall rate of resistance evolution is

$$R \approx u \cdot nN \cdot \frac{1}{\mu - r + d} \cdot \delta s \cdot P_{|F}, \quad (3)$$

where $P_{|F}$ is given by eqn 2.

Note that this model makes the strong hypothesis that clusters are established by a single bacterium and that they keep clonal until extinction, which means that no migrant bacterium can ever establish in a living cluster. Deviations from this hypothesis are likely to occur. For instance, clusters are likely to be established by several bacteria dispersing together. However, in any case this would only increase the genetic polymorphism within each cluster and hence reduce the selective pressure in favour of cooperation, slowing down the evolution of resistance. Therefore, the simplifying hypothesis of clonality is conservative with regard to long-term treatment efficiency. In other words, eqn 3 gives a maximal boundary for the rate of resistance evolution to an anti-cooperative drug.

RESULTS

Let us relate each element of eqn 3 to biological considerations, and compare it with its possible counterpart in the case of conventional antibiotic resistance.

Mutation rate

First, u is the total rate of mutation toward resistance. This rate depends on the per-base mutation rate in the species as well as on the number of different mutations that can lead to resistance. In general terms, this mutation rate u should not differ markedly from the mutation rate toward conventional antibiotic resistance. Note though that the specific use of drugs blocking bacterial communication could have interesting consequences on the amount of constraints exerted on resistance evolution, and therein on the actual mutation rate toward resistance. This issue is discussed at the end of this paper.

Population size

The second factor of eqn 3 is the total bacterial population size nN maintained despite the application of treatment. Therefore, the total number of mutants generated per unit of time is $u \cdot nN$. The population size is likely to be higher when anti-cooperative drugs are used than after conventional antibiotic treatment. Indeed, anti-cooperative treatments do not directly kill bacteria and should therefore be less able than antibiotics to deplete their population size. However, note that certain cooperative traits might be essential for bacterial clusters to reproduce and survive. Their blockade by a treatment could for instance facilitate the clearing action of host's immune system, or reduce the population size of clusters and hence lessen the number of bacteria dispersing to colonize empty patches. In both cases, it could strongly impede bacterial demography.

Further, the number of clusters maintained alive despite treatment (n) affects negatively the probability of fixation after successful dispersal ($P_{|F}$, eqn 2). In consequence, the overall

rate of resistance evolution does not increase indefinitely with n. Obviously, if treatment clears all clusters ($n = 0$), then resistance cannot evolve and $R = 0$. On the contrary, if treatment does not reduce at all the number of living clusters, then resistance does not affect this number either ($n' = n$) and the rate of resistance evolution is nil because resistant clusters have no advantage over sensitive ones (see the expression of $P_{|F}$ in eqn 2). Therefore, the overall rate of resistance evolution reaches a finite maximum for an intermediate depletion of clusters' density ($0 < n < n'$).

Mutants' cumulated longevity

The third factor of eqn 3, the ratio $1/(\mu - r + d)$, does not have an obvious biological meaning. We show in the following that it measures the expected cumulative time the mutant and all its descendants remain in the cluster. Let us consider a resistant mutant appeared in a given cluster. Each mutant is undergoing an overall rate of disappearance from the cluster $\mu + \delta + d$, which is the sum of all sources of disappearance (death and dispersal of the mutant, plus extinction of the whole cluster). Therefore, in expectation, each mutant remains a time $1/(\mu + \delta + d)$ in the cluster and produces a total number of offspring $r/(\mu + \delta + d)$ (we recall that r is the replication rate of mutants). The total number of mutants generated by one independent mutation is then in expectation

$$\sum_{i=0}^{+\infty} \left[\frac{r}{(\mu + \delta + d)}\right]^i.$$

Hence the sum of the longevities of all mutants is

$$T = 1/(\mu + \delta + d) \sum_{i=0}^{+\infty} \left[\frac{r}{(\mu + \delta + d)}\right]^i,$$

which simplifies to $1/(\mu - r + d + \delta)$. Under the hypothesis of weak dispersal T becomes $1/(\mu - r + d)$, which is actually the third factor of eqn 3. If the unit of time is expressed in hour, then this factor represents the actual number of 'mutants·hours' generated in a given cluster following the appearance of a single independent mutation. In other words, each independent mutation will either yield the presence of one mutant for T hours, or the presence of T mutants for 1 h each, or any equivalent combination. The overall probability of resistance fixation is the same in all cases. Therefore, if one considers nN sensitive bacteria, these bacteria are generating an efficient number of 'mutants·hours' equal to $u \cdot nN \cdot T$. In other words, T is relating the microbial mutation rate u to an efficient mutation rate at the scale of the cluster $U = uT$.

The cumulative longevity of mutants, $T = 1/(\mu - r + d)$, actually depends on two distinct features of the system. First, it depends on the strength of local selection against resistance. If resistance is strongly counter-selected in predominantly sensitive clusters then the replication rate of rare mutants is much lower than their death rate ($\mu - r$ is large) and hence T is low. Second, apart from the local cost of resistance, the extinction rate of clusters (d) is also affecting T. However, the extinction rate of clusters cannot be considered as an independent parameter. Indeed, we have assumed that the bacterial population was at a demographic equilibrium hence the extinction rate of clusters must equal their birth rate. We will go back to this important point in the following.

Probability of successful dispersal

The fourth factor of eqn 3, δs, is the probability per unit of time that a given resistant mutant disperses from its original cluster (δ) and successfully establishes a cluster of its own (s). Generally, one can reasonably assume that both resistant and sensitive bacteria have the same dispersal rate δ. The probability for a dispersing mutant to establish a cluster of its own (s) might, however, be larger than for a sensitive individual ($\hat{s}$), because of better colonizing ability

$$s = \hat{s}(1 + \alpha).$$

However, in general, cooperative traits are mostly beneficial when numerous individuals are expressing them together; hence they should barely affect the colonizing ability of bacteria (α should be low). Furthermore, concretely, the anti-cooperative drugs developed so far are blocking bacterial quorum-sensing. Hence, by definition, the cooperative traits they hinder are only expressed once bacteria reach a large density. In consequence, it is likely that these treatments do not affect at all bacteria's ability to colonize new patches ($\alpha = 0$).

Interestingly, the product $b = N\delta\hat{s}$ actually represents the total number of secondary clusters successfully established by any given focal cluster per unit of time. Further, as mentioned above, the assumption of demographic equilibrium implies that the birth rate of clusters equals their extinction rate and therefore that $b = d$. For instance, if clusters have a large extinction rate (large d), then the density of empty patches available for colonization is high also so that dispersing bacteria are more likely to survive and establish new clusters. At equilibrium this exactly compensates and hence $N\delta\hat{s} = b = d$. In consequence, the parameter d does not only represent the extinction rate but, more comprehensively, the turnover rate of clusters, the overall effect of which will be described later on.

Probability of fixation after successful dispersal

The last factor of eqn 3, $P_{|F}$, is the probability of ultimate resistance fixation once a mutant has dispersed from its

original cluster and has successfully established a cluster of its own. Its mathematical expression is given by eqn 2. This factor is typically high if resistance is strongly favourable to entire clusters, i.e. if the treatment strongly depletes bacterial density ($n' \gg n$ in eqn 2). In this case, once an entirely resistant cluster has been founded, then the global rise of resistance is almost a *fait accompli*.

More importantly, this late-acting factor is not what differentiates primarily anti-cooperative and antibiotic resistances, as in both cases resistance is favourable to entire clusters. Therefore, general predictions can hardly be made on the relative value of $P_{|F}$ in both types of treatments. Although $P_{|F}$ depends on the degree to which bacterial population is depleted by treatment (eqn 2). Therefore, it is unlikely for $P_{|F}$ to be generally larger for anti-cooperative treatments than antibiotics, as it would mean that anti-cooperative treatments generally deplete more the number of living bacterial clusters.

Clusters' turnover rate

Let us go back to eqn 3 and express the fact that, owing to demographic equilibrium, clusters' extinction and birth rates are equal ($N\delta\hat{s} = b = d$). Instead of the turnover rate *per se* (d), we consider the parameter $L = 1/d$ representing the expected lifespan of clusters. The rate of resistance evolution can then be rewritten

$$R \approx u \cdot n \cdot \frac{1+\alpha}{1+(\mu - r)L} \cdot P_{|F}. \quad (4)$$

The clusters' lifespan controls the importance of local selection ($\mu - r$) relative to the hazard of transmission. Each cluster's reproduction event implies a strong bottleneck as clusters are established by a single bacterium. Therefore, if clusters die and reproduce often (high turnover rate and thus low L) then local selection becomes too weak to affect significantly the fate of mutants [$(\mu - r)L \ll 1$ in eqn 4]. Because resistance is cooperative it is locally deleterious ($r < \mu$). Therefore, the rate of resistance evolution is decreasing with clusters' lifespan. This observation, allowed by the simplicity of eqn 4, is confirmed in Fig. 2a, both from stochastic simulations, and from the evaluation of the rate of resistance evolution in the general case (i.e. not assuming weak dispersal). The same result is obtained under the assumption that treatment yields the complete eradication of all bacteria (see Appendix S3).

The advantage of targeting cooperation

Let us assume that resistance does not affect the colonizing ability of bacteria ($\alpha = 0$), and recall that $P_{|F}$ being a probability it is lower than one. From eqn 4 and considering the fact that resistance is cooperative and hence locally deleterious ($r < \mu$), the rate of resistance evolution obeys the inequality $R \leq u \cdot n$, where we recall that u is the microbial mutation rate towards resistance and n is the total number of clusters maintained alive despite treatment. Surprisingly, in a situation where a total of $N_T = nN$ bacteria are maintained alive despite treatment and can potentially generate resistance at each time step,

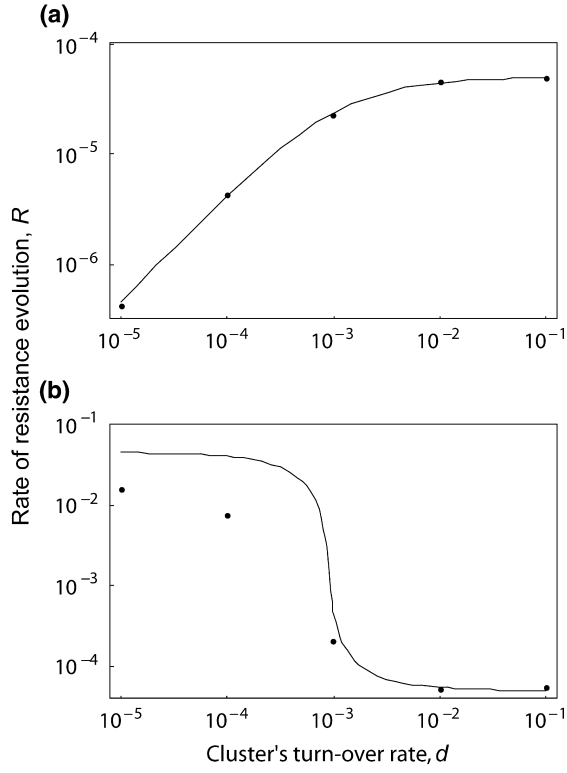

Figure 2 Rate of resistance evolution as a function of the turnover rate of clusters. Dots are results of stochastic simulations, based on the estimate of the probability of fixation of resistant mutants averaged over 1000 fixations (see Appendix S4). Lines are exact results from the branching process model (eqn A1). In (a), resistance is counter-selected by within-cluster competition. Within a predominantly sensitive cluster, the replication, death, and dispersal rates of resistant mutants are $r = 10^{-3}$, $\mu = 2.10^{-3}$ and $\delta = 10^{-4}$ respectively. The mutation rate towards resistance is $u = 10^{-6}$; the number of clusters is $n = 100$ and the number of bacteria per cluster $N = 10^3$. The effect of resistance on colonizing ability is nil ($\alpha = 0$). The probability of ultimate resistance fixation once a mutant has successfully established a cluster of its own is $P_{|F} = 0.5$. In (b) resistance is favoured by within-cluster competition. All parameters are as in (a) except the within-cluster replication and death rates of resistant mutants, which are $r = 2.10^{-3}$ and $\mu = 10^{-3}$ respectively. When clusters go extinct very often (large turnover rate), then local selection has a negligible impact on the rate of resistance evolution (see eqn 4), which tend towards $R \approx u \cdot n \cdot (1 + \alpha) \cdot P_{|F} = 5 \times 10^{-5}$ in both (a) and (b).

we end up with an actual rate of resistance evolution relying only on the number of clusters, n. An order of magnitude N has been gained (where N is the number of bacteria per cluster). In comparison, if a total of nN bacteria were to be treated by an antibiotic, the likelihood of resistance evolution would rely on the nN individuals and not on the number of clusters (n). Note that this advantage of anti-cooperative treatments relative to antibiotics is also found under the assumption that treatment yields eradication (see Appendix S3).

In order to illustrate this result, one can rewrite the rate of resistance evolution as

$$R \approx n \times b \times U \times P_{|F},$$

which is the product of (i) the number of clusters n, (ii) the reproduction rate of clusters $b = \delta N\hat{s}$, (iii) the probability for each secondary established cluster to be a mutant $U = uT$ and (iv) the probability for each mutant cluster to generate global resistance $P_{|F}$. Each one of these factors is regarding entire clusters and no longer individual bacteria. The number of microscopic bacteria per cluster is included into the macroscopic birth rate of clusters. The microscopic properties of bacteria (replication and mortality rates) are included in the parameter T. Finally, the parameter T relates the mutation rate at the microscopic level (u) to a macroscopic mutation rate (U).

Clusters' size

The total number of bacteria kept alive, despite the use of a given treatment, $N_T = nN$, is taken as the measure of the therapeutic efficiency of that treatment. Introducing this parameter into the above inequality and assuming that cooperation does not affect colonization ($\alpha = 0$) yields to a novel inequality

$$R \leq u \cdot \frac{N_T}{N}.$$

Both for medical purposes, and in order to minimize the likelihood of resistance evolution, treatments should deplete bacterial populations (decrease N_T). The potential reduction of bacterial population size, i.e. the therapeutic success of treatment, might however be limited for various reasons. The major interest of the present approach is then to compare various treatments with the same therapeutic benefits. Here we show that, for a given ability to deplete bacterial demography (given N_T), resistance evolution is less likely if the size of each cluster (N) is large. This is again because of the fact that clusters, and not bacteria, are the actual units generating resistance. Therefore, if each cluster is large then the number of units generating resistance is low, which reduces the likelihood of resistance evolution. Also, here the same result is obtained under the assumption that treatment yields eradication (see Appendix S3).

Local counter-selection vs. local advantage

The present model considers specifically the case of treatments targeting cooperative traits. In this case resistant mutants are counter-selected locally, which is expressed mathematically through the fact that their replication rate is lower than their death rate ($r < \mu$). Further, the technique employed to build the model makes the central assumption that resistant mutants do not reach a significant frequency within their original cluster, which implies that they are counter-selected locally.

However, it is possible that the local cost of resistance is not that strong ($\mu \approx r$). With different types of treatments it is even possible that resistance is slightly advantageous within clusters ($\mu < r$). Interestingly, our model can also provide some insights into these cases. If clusters most often go extinct as a whole shortly after the appearance of resistant mutants (low $L = 1/d$), then even neutral or slightly advantageous mutants are highly unlikely to ever reach a significant frequency within their cluster. Furthermore, in bacteria (or viruses) clusters should typically contain a large number of individuals, and it should take numerous generations for advantageous mutants to grow to a significant frequency. The model therefore remains valid even when resistance is locally neutral or slightly advantageous, provided that clusters' turnover rate is large enough (compare simulations and analytic results in Fig. 2b). In this case, in contrast with the case where resistance is counter-selected, resistance evolves more slowly when clusters have a short lifespan because local selection is then less efficient (see eqn 4 and Fig. 2b). Further, if clusters have a very short lifespan, then the rate of resistance evolution is of the same order of magnitude than for locally counter-selected resistance (close to $u \cdot n$, see Fig. 2b).

In general terms, when local selection is weak and/or clusters' lifespan is short, then mutants become effectively neutral locally [$(\mu - r)L \ll 1$ in eqn 4]. Bottlenecks, occurring at the foundation of novel clusters, are so frequent that local selection is not strong enough to affect significantly the fate of mutants. This finding brings an important question. If local selection is less considered, then what differentiates resistance to anti-cooperative treatments from conventional antibiotic resistance? Indeed, the key difference between both is supposed to reside in their local properties: anti-cooperative resistance is locally disfavoured while antibiotic resistance is locally favoured. However, some other features might still differentiate the two. First, it is likely that antibiotic resistance is not only slightly favoured locally but very strongly favoured. Therefore, the lifespan of clusters should be unrealistically short for the local

advantage of resistance to become inefficient. Second, and more importantly, in numerous cases, the brief lifespan of clusters can be directly explained by their sensitivity to treatment. In the case of antibiotics, it follows that the mere appearance of a resistant mutant within a cluster increases its lifespan by preventing eradication. In contrast, in the case of anti-cooperative treatments, resistance becomes efficient only once a significant proportion of the cluster is expressing it. Therefore, the presence of rare mutants within a cluster does not compromise the efficiency of treatment, and the cluster's probability of extinction remains high. Apart from the local counter-selection of resistance, this effect might be another important feature of anti-cooperative treatments slowing down the evolution of resistance.

DISCUSSION

The objective of anti-bacterial treatments is to get rid of infections. In this aim, antibiotics target traits vital for each individual microbe and this harms the infection. Accordingly, being resistant to antibiotics is advantageous for each individual in the competition with others, and resistance can be rapidly selected for, among the important variability generated by bacteria. In this paper, we have considered an alternate type of anti-infectious treatments targeting traits defined as cooperative, i.e. essential for groups of bacteria called clusters, but costly to express for each individual. Accordingly, being resistant as a whole is advantageous for clusters, but it is not advantageous for individual bacteria. A concrete example may help understanding. Certain drugs can block the communication system of bacteria, which prevents the secretion of virulence factors (see Williams *et al.* 2000). The lack of virulence factors is costly for bacterial clusters as these factors are necessary for efficient host exploitation. Therefore, when treatment is used, resistant clusters are favoured over sensitive ones. However, within a predominantly sensitive cluster, resistant individuals are counter-selected, as the virulence factors they secrete are shared and do not benefit more to themselves than to their sensitive neighbours. In brief, resistance is favourable once fixed within a cluster, but counter-selected within clusters.

Note that these alternate treatments might have slightly different roles than conventional drugs. Indeed, in contrast with antibiotics, anti-cooperative treatments do not kill individual bacteria but disorganize bacterial groups. This might have drastic consequences on infections if the coordinate regulation of bacterial behaviour is required for instance to resist host-immune system. However, in general terms, anti-cooperative treatments are likely to reduce bacterial density to a lesser extent than antibiotics. In other words, while antibiotics aim at eradicating, anti-cooperative treatments might mostly aim at controlling infections, which could certainly prove to be useful in certain but not all situations.

However, the effect of treatments on the symptoms of infections is not the subject of the present analysis. We are interested in a different aspect of anti-cooperative treatments, namely their properties with regard to resistance evolution. Interestingly, when treatment blocks cooperation, resistance to that treatment is by definition cooperative as it resumes cooperation. The aim of the present paper was to analyse mathematically the consequence of this peculiarity on the rate of resistance evolution.

We have shown that the likelihood of resistance evolution against anti-cooperative drugs is only proportional to the total number of clusters in the population (n) and is not depending on the total number of bacteria (nN). This is explained as follows. In the first stages of their existence, resistance mutations are experiencing the strong cost of cooperation (e.g. producing virulence factors) without receiving the beneficial counterparts from neighbours. In order to benefit from retrieved cooperation and generate global resistance, resistant mutants must disperse from their original cluster and establish a cluster of their own in an available empty patch. In consequence, the emergence of resistance is really depending on the reproduction of clusters and not on the reproduction of bacteria. As an appealing interpretation one can say that by targeting treatments against adaptive properties of groups instead of individuals, we shift one level up the relevant unit of organization generating resistance. Resistance is then evolving at a slower pace, because groups are less numerous than individuals ($n < nN$). In other words, with anti-cooperative drugs, instead of facing billions of bacteria we face a reduced number of larger organisms (clusters) with lower evolutionary potential. A key element of the success of treatment is therefore the size of each cluster. Treatments should target cooperative traits for which clusters are as large as possible so that, for a given number of bacteria, the number of clusters is lower.

What may then be the size of clusters in various instances? With regard to the application of a treatment, a cluster represents the minimal number of neighbouring microbes that must be resistant for their resistance to become beneficial. This critical mass depends on the adaptive trait targeted by treatment. At one extreme, in the case of conventional antibiotics each individual is a cluster: resistance is favoured even when expressed by a single bacterium. In consequence antibiotic resistance appears and fixes very rapidly, because the number of clusters n is very large (n is actually the total number of bacteria). At the other extreme treatments can target microbial features that are beneficial for the entire infection within a host. Numerous viruses, for instance, inhibit inflammatory or immunological responses by

interfering with host's cytokines (see Bonhoeffer & Nowak 1994). If a treatment blocks such a cooperative trait, then resistant individuals are always disfavoured within the host as they are the only ones to exert a costly manipulation to the benefit of the entire infection. Examples of such traits in bacteria could include the secretion of virulence factors (e.g. Brown & Johnstone 2001). For instance, *Pseudomonas aeruginosa* secretes siderophores making host's iron accessible to bacteria (see West & Buckling 2003). If the concentration of accessible iron experienced by each bacterium depends upon the average siderophores secretion over the infection (i.e. siderophores are shared by the entire infection), then sensitive individuals (not secreting siderophores) will always be favoured over resistant ones. In all these cases, clusters are as big as they can be as they represent the entire infectious populations within hosts. However, we believe that most bacterial virulence factors (e.g. siderophores) are likely to be favoured at an intermediate level, a neighbourhood of bacteria within the infection. Once such a neighbourhood is entirely resistant to treatment (secretes virulence factors), the secreted factors provide it with an advantage over sensitive neighbourhoods, and resistance invades the infection. A comprehensive analysis of this case would require building a spatial model of population, with isolation by distance instead of cluster-structure (e.g. van Baalen & Rand 1998). In general, let us simply underline that, given its importance for resistance evolution, it is an important empirical perspective to characterize the relevant cluster size for various infectious traits that could be potential targets for future anti-cooperative treatments.

We have so far analysed generally the evolution of resistance to any anti-cooperative treatment. In practice, although, most approaches to target bacterial cooperation precisely aim at interfering with the control of gene expression by quorum-sensing (Eberhard *et al.* 1986; Passador *et al.* 1996; Schaefer *et al.* 1996; McClean *et al.* 1997; Swift *et al.* 1997, 1999; Balaban *et al.* 1998; Finch *et al.* 1998; Mayville *et al.* 1999; Williams *et al.* 2000; Alksne 2002; see also Koerber *et al.* 2002 for a theoretical approach). Here, we discuss briefly the potential mechanisms of action of such quorum-sensing blocking drugs, and suggest some potential implications for resistance evolution. Recall that quorum-sensing is a two-component communication system. Each bacterium secretes a diffusible signal and expresses a corresponding receptor. When the concentration of signals measured by receptors exceeds a threshold, virulence factors are produced.

Regardless of the precise mechanism for quorum-sensing blockade, bacteria really have two ways to evolve resistance. First, they can reinstate quorum-sensing and the proper regulation of gene expression. Second, they can give up communication, and evolve towards the constitutive expression of virulence factors. Interestingly, the analysis developed in the present paper applies equally to both cases, because resistant individuals secrete proteins to the benefit of their whole group and are therefore necessarily cooperative.

Yet, the two resistant mechanisms are very different. It is indeed plausible that the coordinate secretion of virulence factors, and not merely their presence, determines the success of bacterial groups. Therefore, constitutive gene expression is probably a rough response of bacteria to treatment, and the restoration of proper communication could be a more general resistance mechanism. Let us analyse briefly the way bacteria can restore communication in the presence of a treatment. Any treatment can be considered as a lure, mimicking a functional protein in order to bind another and prevent a functional relationship to establish. In the precise case of quorum-sensing blockade, potential treatments are of two types. They can either mimic the signal molecule to bind receptors and prevent them from sensing the actual signal, or on the contrary, mimic the receptor to bind signal molecules before they reach bacterial cells and/or accelerate their degradation (see Koerber *et al.* 2002). In each case, such as with antibiotics, resistance involves mutations that reduce the affinity of the target for the lure, without reducing too much its affinity for the corresponding functional protein (see Andersson & Levin 1999 in the case of antibiotics). Therefore, resistance is less likely to evolve if the drug mimics in an accurate way the functional protein. However, constraints exist on the degree of resemblance between drugs and their bacterial counter-parts, most importantly because drugs must not form functional relationships with their target. For instance, if a drug mimics the quorum-sensing signal, then it must be able to bind receptors but, unlike the signal itself, it must not activate them. Interestingly, in the case of a drug mimicking the quorum-sensing receptor, this constraint is less of a matter. As long as the decoy receptor is in a soluble form with no functional relationship with bacteria, it is unable to respond functionally to the signal. As a result, it might be possible to use very close analogues of the receptor, or even the receptor itself, as a treatment. Very few mutations could then lead to viable resistance, and the efficient mutation rate towards resistance would be very low. Ideally, the restoration of bacterial communication would require the co-evolution of the two quorum-sensing components, which would be slower than the evolution of a single protein. So far, most attempts to block quorum-sensing have used analogues of the signal (see Williams *et al.* 2000). The present argument suggests that the use of analogues of the receptor might also be a

valuable pathway of research. In general terms, we want to point out that, apart from the cooperative nature of resistance discussed above, interfering with an extracellular signalling pathway such as quorum-sensing, instead of an intracellular mechanism, might have interesting consequences for resistance evolution.

In conclusion, this work suggests that the multicellular aspects of microbial infections could be fruitfully exploited in the design of treatments. Metazoans can be killed by simple disorganizations of their multicellular activities (e.g. through hormones dysfunction, cancer, etc.), or by the destruction of very few specialized cells (e.g. neurones and myocardic cells). The destruction of a whole metazoan certainly does not imply the one-by-one destruction of each of its individual cells. In fact, the cellular differentiation and fine regulation of intercellular relationships in metazoans is altogether the core of their success and complexity but also their Achilles' heel to a large extent. Here we suggest that, to a lower degree, it might be true also in certain microbes, and that among the multicellular features of microbial infections Achilles' heel might be found and used as valuable drug targets.

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SUPPLEMENTARY MATERIAL

The following supplementary material is available online for this article from http://www.Blackwell-Synergy.com:

Appendix S1 Probability for a resistant mutant to emerge
Appendix S2 Fixation probability after cluster foundation, $P|_F$
Appendix S3 Escape probability
Appendix S4 Simulations

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Appendix S1 Probability for a resistant mutant to emerge

Here we derive an approximate expression of the probability P for a resistant mutant to generate global resistance. Equation 1 of text is solved to yield

$$P = 1 - K = (1/2r)\left[\sqrt{(\mu - r + \delta + d)^2 + 4r\delta s P_{|F}} - (\mu - r + \delta + d)\right]. \quad \text{(A1)}$$

Developing P into a Taylor series around $\delta = 0$ yields

$$P = P|_{\delta=0} + \delta \cdot \frac{\partial P}{\partial \delta}|_{\delta=0} + o(\delta),$$

which gives the approximation for P given in the text.

Appendix S2 Fixation probability after cluster foundation, $P_{|F}$

This probability is calculated by modelling the demography of resistant clusters as a continuous-time model (see also Iwasa *et al.* 2004). Consider an entirely resistant cluster present in the meta-population at time t, and define $K_{|F}(t)$ as the probability that all the resistant mutants present in this cluster are ultimately lost. The resistant cluster dies at a constant rate d' owing to catastrophic extinction, and generates secondary clusters by transmission to empty patches at a rate b'. As a result, $K_{|F}(t)$ can be expressed by considering the events potentially occurring during an infinitesimal period:

$$K_{|F}(t) = b'\mathrm{d}t(K_{|F}(t + \mathrm{d}t))^2 + d'\mathrm{d}t + (1 - d'\mathrm{d}t - b'\mathrm{d}t)K_{|F}(t + \mathrm{d}t). \quad \text{(A2)}$$

Assuming that resistant clusters are strongly favoured, the fixation of resistance becomes deterministic as soon as a small number of clusters are actually resistant (see main text). As a result, during the whole stochastic process, sensitive clusters remain largely predominant in the meta-population. Following a mass-action model, the birth rate of resistant clusters can then be written as $b' = \beta' h$, the product of a transmissibility coefficient (β'), which is an intrinsic property of resistant clusters, by the density of empty sites (h), an ecological property of the meta-population. Assuming that sensitive clusters remain predominant during the stochastic process means that the number of free sites available for transmission remains solely controlled by sensitive bacteria and is thus constant through time. In consequence, the birth rate b' of resistant clusters is constant through time, and the probability of loss after foundation, $K_{|F}(t)$, can be considered as constant as well. This leads to a simplified version of eqn A2:

$$b'K_{|F}^2 - (d'\mathrm{d}t + b'\mathrm{d}t)K_{|F} + d' = 0,$$

which can be solved to yield an expression for $P_{|F} = 1 - K_{|F}$:

$$P_{|F} = 1 - \frac{d'}{b'}. \quad \text{(A3)}$$

Note that this expression is equivalent to the probability of escape obtained by Iwasa *et al.* (2004). Recall that the birth rate of resistant clusters can be written as $b' = \beta' h$. Further, if resistance happens to fix, bacteria will attain a novel demographic equilibrium where the extinction rate of clusters equals their birth rate. Therefore, one can express the extinction rate of clusters as $d' = \beta' h'$, where h' is the equilibrium density of empty sites once resistance is fixed in the meta-population. As a result, the probability of fixation can be re-written as

$$P_{|F} = 1 - \frac{h'}{h}.$$

The densities of available empty sites, h and h', can finally be expressed as a function of the total number of available sites, S, to yield eqn 2 of text.

Appendix S3 Escape probability

In the main text, the treatment is unable to yield the complete eradication of bacteria. Here we develop an alternate model where, on the contrary, treatment yields the ultimate extinction of all bacteria except if resistance is generated rapidly enough. After treatment, the bacterial density is varying (decreasing) through time. At any time step t, this shrinking population generates resistance at a given rate $R(t)$. Therefore, rather than calculating the rates of resistance evolution at every instant, we integrate their effects through time by calculating the overall probability that the initial bacterial population ultimately generates resistance before extinction (the probability of escape, *sensu* Iwasa *et al.* 2004).

Precisely, we assume that treatment does not lead to the destruction of each cluster *per se*. After application of the drug, each cluster reaches rapidly a new equilibrium size with N bacteria. However, the reproduction rate of these clusters is not sufficient to compensate for their frequent extinctions; therefore, the number of present clusters decreases indefinitely from n_0 at the initiation of treatment down to zero.

The demography of each cluster being stabilized, with small variations around a density of N bacteria per cluster, the replication and death rates of each sensitive bacterium can be considered as constants and equal to each other ($\hat{\mu} = \hat{r}$, where the hat refers to sensitive

individuals). However, the number of clusters is gradually decreasing, because clusters go extinct at a rate d larger than their birth rate b. Considering that the treatment strongly impedes the demography of clusters, we assume that the two demographic parameters b and d are independent of the actual number of clusters present at a given time, and are hence constant through time. Consider then a given sensitive bacterium present at time t in a focal cluster. The probability that this bacterium and all its descendants disappear without generating resistance (the probability of ultimate loss of the bacterium) is the same than the probability of ultimate loss of any other bacterium taken at any other time in the population. This probability is called q and is hence the solution of the following equation

$$rq^2 + \mu + d + \delta(1-\hat{s}) + \delta\hat{s}q^N + u(1-P) - (r+\mu+d+\delta+u)q = 0, \quad \text{(A4)}$$

where u is the mutation rate towards resistance, and where we recall that P is the probability for a resistant individual to generate global resistance (escape). The product $\delta\hat{s}q^N$ is the probability that the focal sensitive individual disperses from its cluster (probability δ), succeeds in establishing its own cluster of size N (probability $\hat{s}$), and is finally lost because all the individuals of the established cluster are lost (probability q to the power N). However, this power N of q is simplified. We assume that the probability for a focal sensitive bacterium to generate escape is very low ($p = 1 - q \approx 0$). Therefore $\delta\hat{s}q^N$ can be simplified to the first order in p giving $\delta\hat{s}(1 - Np)$. In order to be consistent, the term rq^2 is also taken to first order in p giving $r(1 - 2p)$. Note that the exact same result is obtained if the two terms are taken to the second order in p. Equation A4 can then be solved to find an analytical expression for $p = 1 - q$ (not shown). This solution is then developed into a Taylor series to the first order of mutation rate, which is reasonable as mutation rates are usually low. Finally we use the fact that $\mu = \hat{r}$ which gives

$$p = \frac{uP}{(d + \delta - N\delta\hat{s})}.$$

Considering then the whole bacterial population at time 0 with n_0 clusters of size N, the probability that at least one bacterium generates escape is

$$P_e = 1 - (1-p)^{n_0 N}.$$

We finally assume that the probability that more than one bacterium generates escape is negligible ($n_0 NP \ll 1$), which yields $P_e \approx n_0 Np$ leading to

$$P_e = [un_0 N/(d + \delta - N\delta\hat{s})] \cdot P.$$

In the case of weak dispersal, with the formulae for P given by eqn A1, this gives

$$P_e = u \cdot n_0 \frac{N\delta s}{d + \delta - N\delta\hat{s}} \cdot \frac{1}{\mu - r + d} \cdot P_{|F}, \quad \text{(A5)}$$

where $\hat{s}$ is the probability for sensitive bacteria to survive in the external environment and establish a cluster by their own, and $P_{|F}$ is given by eqn 2. Equation A5 assumes that both sensitive and resistant individuals have the same dispersal rate δ.

Comparing escape probability and rate of evolution

Equation A5 can be rewritten as

$$P_e = u \cdot n_0 \left[b\frac{(1+\alpha)}{(d-b)} \right] \cdot \left[\frac{1}{(\mu - r + d)} \right] \cdot P_{|F},$$

where we recall that $b = N\delta\hat{s}$ is the birth rate of sensitive clusters and d their extinction rate. On the contrary, the rate of resistance evolution can be rewritten from eqn 3 as

$$R = u \cdot nb(1+\alpha) \cdot \left[\frac{1}{(\mu - r + d)} \right] \cdot P_{|F}.$$

For better illustration, consider the simple case where resistance does not confer any colonizing advantage to bacteria ($\alpha = 0$). The rate of evolution depends on the product nb, which represents the expected total number of secondary clusters founded in the population, per unit of time. In the probability of escape, this term is replaced by the ratio $n_0 b/(d - b)$, which can be shown to represent the expected total number of secondary clusters founded in the population, from the initiation of treatment until extinction of all bacteria. When cluster density is assumed as constant, then we derive a constant rate of resistance evolution, and the key parameter is the rate of cluster reproduction. Whereas, when cluster density is shrinking down to zero, then we derive an overall escape probability, and the key parameter is the total number of cluster reproduction events. Note that in the general case ($\alpha \geq 0$), the two quantities must simply be multiplied by the colonizing ability of resistant individuals relative to sensitive individuals ($1 + \alpha$).

The advantage of targeting cooperation

In the main text we show that, due to local counter-selection, the rate of resistance evolution is lower than a threshold ($R \leq u \cdot n$). An equivalent inequality can also be derived for the escape probability. Indeed, when the number of clusters is shrinking owing to treatment, their birth rate is lower than their death rate ($b < d$). As a result, from eqn A5, the escape probability obeys the inequality

$$P_e < u \cdot \left[\frac{n_0}{(d-b)} \right].$$

This last formula differs in a simple way from its rate-of-evolution equivalent. The number of clusters present at any given time (n) is simply replaced by the total number of clusters generated from the initiation of treatment until extinction [$n_0/(d - b)$].

Therapeutic efficiency

In the rate-of-evolution model the therapeutic success of a treatment was measured by the number of bacteria kept alive despite treatment ($N_T = nN$). In the probability-of-escape model, therapeutic success can be measured by the same parameter. One can integrate through time the number of bacteria present in the population at each time step, from the first application of treatment until complete eradication; this gives

$$\Sigma N_T = \frac{n_0 N}{(d - b)}.$$

This sum is a time-integrated analogous of $N_T = nN$ when the number of clusters (n) is varying through time. The escape probability is therefore following the inequality $P_e < u \cdot \Sigma N_T/N$, where N is the number of bacteria per cluster. Therefore, here also, for a given therapeutic efficiency (given ΣN_T), the risk of treatment failure is lower if clusters are large (large N). This is because here also the evolution of resistance is relying on clusters and not bacteria.

Appendix S4 Simulations

Monte-Carlo simulations are performed. Simulations are initiated with a single mutant bacterium, appearing inside a treated cluster infected by sensitive bacteria. At each step of the model, one event is chosen randomly among four: (i) extinction of the cluster, (ii) replication of one of the present mutants, (iii) death of a mutant or (iv) dispersal of a mutant. Each event is chosen according to its rate of occurrence. Once in the external environment, dispersing mutants have a fixed probability s to generate a resistant cluster by their own. The replication rate of mutants is calculated at each step of the model and varies as a function of their density inside the cluster. Therefore, the simulations relax the branching hypothesis (constant life-history traits of mutants). At one extreme, if mutants are rare, their replication rate is constant and depends solely on their competitive ability relative to sensitive bacteria. At the other extreme if mutants are fixed within the cluster, their replication rate must be equal to their death rate plus their dispersal rate (the total cluster size is at equilibrium). This effect is represented by a linear relationship between the number of mutants in the cluster and their replication rate:

$$r(t) = r_0 + \frac{\mu + \delta - r_0}{N} x(t), \tag{A6}$$

where $x(t)$ is the number of mutants present in the cluster at time t and N is a fixed parameter representing population size. The mutant's mortality and dispersal rates are assumed constant. We perform simulation runs starting from a single mutant; we measure the proportion of runs where the mutant ultimately generates a resistant cluster by its own; this should be equal to the probability calculated analytically. The overall rate of resistance evolution is then calculated analytically by multiplying the observed probability of fixation by unN, where u is an arbitrary mutation rate, n is the number of clusters and N is the number of bacteria per cluster.