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Understanding policing as a mechanism of cheater control in cooperating bacteria

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Abstract

Policing occurs in insect, animal and human societies, where it evolved as a mechanism maintaining cooperation. Recently, it has been suggested that policing might even be relevant in enforcing cooperation in much simpler organisms such as bacteria. Here, we used individual-based modelling to develop an evolutionary concept for policing in bacteria, and identify the conditions under which it can be adaptive. We modelled interactions between cooperators, producing a beneficial public good, cheaters exploiting the public good without contributing to it, and public good producing policers that secrete a toxin to selectively target cheaters. We found that toxin-mediated policing is favoured when (i) toxins are potent and durable, (ii) cheap to produce, (iii) cell and public good diffusion is intermediate, and (iv) toxins diffuse farther than the public good. While our simulations identify the parameter space where toxin-mediated policing can evolve, we further found that policing decays when the genetic linkage between public good and toxin production breaks. This is because policing is itself a public good, offering protection to toxin-resistant mutants that still produce public goods, yet no longer invest in toxins. Our work thus highlights that not only specific environmental conditions are required for toxin-mediated policing to evolve, but also strong genetic linkage between the expression of public goods, toxins and toxin resistance is essential for this mechanism to remain evolutionarily stable in the long run.

Keywords

individual-based simulation; public goods dilemma; evolution of cooperation; cheater control mechanism; sociomicrobiology

Introduction

Cooperation, a process where individuals act to increase the fitness of others at an immediate cost to themselves, is common across the tree of life (Sachs *et al.*, 2004, West *et al.*, 2007b).

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Data archiving

The data associated with this study will be archived on Dryad upon acceptance of the manuscript.

While cooperation is widespread in humans (Fehr & Fischbacher, 2003) and other animals such as insects and vertebrates (Clutton-Brock, 2002, Ratnieks *et al.*, 2006), we have only recently recognized that microbes also evolved adaptive cooperative behavior (West *et al.*, 2007a, Nadell *et al.*, 2009, Ross-Gillespie & Kümmerli, 2014, Cavaliere *et al.*, 2017). Types of microbial cooperation include the formation of biofilms, where individuals secrete polymeric compounds to build a protective extracellular matrix (Nadell *et al.*, 2009), the formation of fruiting bodies where some individuals sacrifice themselves to enable the dispersal of others (Velicer & Vos, 2009); and the secretion of shareable metabolites, including proteases to digest nutrients (Diggle *et al.*, 2007, Sandoz *et al.*, 2007), siderophores to scavenge extra-cellular iron (Griffin *et al.*, 2004, Kümmerli *et al.*, 2010), and biosurfactants enabling group motility (de Vargas Roditi *et al.*, 2013).

Although cooperation is thought to provide benefits for the collective as a whole, it is intrinsically vulnerable to exploitation by cheating mutants, which benefit from the cooperative acts performed by others, but refrain from contributing themselves to the welfare of the group (Ghoul *et al.*, 2014). Thus, there has been great interest in identifying mechanisms that maintain cooperation and prevent the invasion of cheaters (West *et al.*, 2007b, Asfahl & Schuster, 2017, Özkaya *et al.*, 2017). Work on microbes have proved particularly useful in this context because cheating mutants can easily be engineered and factors important for cooperation can be experimentally manipulated in laboratory settings. Studies following this approach revealed a plethora of mechanisms promoting cooperation, including: (i) limited dispersal ensuring that cooperators stay together (van Gestel *et al.*, 2014, Kümmerli *et al.*, 2009a); (ii) molecular mechanisms allowing the recognition of other cooperators (Mehdiabadi *et al.*, 2006, Smukalla *et al.*, 2008, Rendueles *et al.*, 2015); (iii) regulatory linkage between multiple traits imposing additional costs on cheaters (Jousset *et al.*, 2009, Dandekar *et al.*, 2012, Ross-Gillespie *et al.*, 2015, Mitri & Foster, 2016, Popat *et al.*, 2017); and (iv) mechanisms reducing the cost of cooperation such as public good recycling (Kümmerli & Brown, 2010) or the use of superfluous nutrients for public goods production (Xavier *et al.*, 2011, Sexton & Schuster, 2017).

In addition, several studies suggested that bacteria have evolved policing mechanisms, which enable cooperators to directly sanction cheaters and thereby enforce cooperation (Manhes & Velicer, 2011, Inglis *et al.*, 2014, Wang *et al.*, 2015, Majerczyk *et al.*, 2016, Evans *et al.*, 2018). For example, Wang *et al.* (2015) showed that in the opportunistic pathogen *Pseudomonas aeruginosa*, the synthesis of a publicly shareable protease is regulatorily coupled to the synthesis of the toxin cyanide in such a way that cheaters, deficient for protease production, also no longer produce the toxin and lose the immunity against it. Thus, the costly synthesis of a harmful toxin, which selectively targets non-cooperative cheaters, can be understood as a policing mechanism. While it seems intriguing that organisms as simple as bacteria can perform policing behavior, multiple questions regarding the evolution of microbial policing have remained unaddressed. For one thing, we know little about the environmental conditions promoting policing via toxin production. In this context, one would expect that the spatial structure of the environment, which affects the diffusion of cells, public goods and toxins, should play a crucial role (Driscoll & Pepper, 2010, Inglis *et al.*, 2011, Dobay *et al.*, 2014). Moreover, it is unknown how potent a toxin must be and how much it can cost in order to efficiently fight cheaters. Finally, it remains unclear whether

policing is an evolutionarily stable strategy or whether non-policing cooperators, which are immune against toxins, can exploit policers as second order cheaters (Inglis *et al.*, 2014), as it is the case in animals (Boyd *et al.*, 2003).

Here, we address these issues by using realistic individual-based models that simulate interactions between cooperating, policing and cheating bacteria on a toroidal two-dimensional surface (Dobay *et al.*, 2014). Individual- (or agent-based) models offer the advantage to study collective behaviours emerging from stochastic processes, guided by dispersal of individuals and random diffusion of molecules on a continuous landscape as it would occur in natural settings. In our *in-silico* approach, bacteria are modelled as discs and seeded in low numbers onto the surface of their habitat, where they can consume resources, grow, divide, disperse, and secrete compounds according to specified parameters. Important to note is that both bacteria and public good molecules are modelled as individual agents and are free to diffuse on a continuous landscape, closely mimicking biological conditions (Figs. 1 and S1). For our main analysis, we considered two bacterial strains that match those used in the empirical policing paper by Wang *et al.* (2015). Specifically, we implemented a policing strain *P* that produces a public good together with a tightly linked toxin and its immunity mechanism; and a cheating strain *C* that is deficient for public good production and, due to the regulatory linkage, is also unable to express the toxin and the corresponding immunity mechanism. We further considered a cooperating control wildtype strain *W*, which produces the public good but features no policing mechanism. Finally, we also considered a second-order policing cheater *R*, which produces the public good, expresses the immunity mechanism, but not the toxin. For this strain, we assume that the genetic linkage between the three traits can be broken, such that *R* directly evolves from *P*.

In a first set of simulations, we examined the performance of the cooperator, cheater and policing strains in monoculture to understand how the relative costs and benefits of public good and toxin production affect strain fitness. Next, we competed wildtype cooperators against cheaters across a range of bacterial and public good diffusion coefficients to determine the parameter space where cooperation is favoured in the absence of policing (Dobay *et al.*, 2014). Subsequently, we competed the cheater against the policing strain across the same parameter space, to test whether policing extends the range of conditions across which cooperation is favoured, and to identify properties of the toxin system (diffusion, potency, durability) to guarantee efficient policing. Finally, we simulated the situation where toxin-resistant public good producers evolve from the policing strain, and ask whether policing via toxin production itself constitutes an exploitable public good.

Materials and methods

In-Silico Habitat and Bacteria

The simulation platform was initially created by Dobay *et al.* (2014). Here, we developed it further and customized it for the purpose of our study. In brief, the *in silico* habitat consists of a two-dimensional continuous toroidal surface, with no boundaries. The size of the surface is $60 \times 60 \mu\text{m}^2 = 3,600 \mu\text{m}^2$. Bacteria are modelled as discs with an initial radius of $0.5 \mu\text{m}$. Bacteria can consume resources, grow at a basic growth rate μ , and divide when reaching the threshold radius of $1.0 \mu\text{m}$. Resources allowing basic growth were unlimited in

our system to ensure that differences in growth patterns emerge solely due to public goods, toxins and social interaction between strains. Bacteria can disperse on the landscape according to a specific cell diffusion coefficient D_c ($\mu\text{m}^2/\text{s}$), and are not bound to a grid, but can freely move on the continuous landscape (i.e. we used an off-lattice model with double-precision floating-point format). At the beginning of a simulation, we randomly seed one founder bacteria of each strain onto the surface, free to grow and divide according to its life cycle (see Figs. 1 and S1 for a visualization).

In addition to the basic growth rate μ , the growth of a bacterium is influenced by its social behavior and its interaction with other community members. Costs, reducing growth, incur to individuals producing public goods, toxins and expressing the immunity mechanism. Additional costs incur to susceptible cells taking up toxins. The uptake of a public good, meanwhile, has a positive effect on growth for the beneficiary. Accordingly, the growth of our four strains W (public good producing wildtype strain), P (policing strain), C (toxin-sensitive cheating strain), and R (second-order toxin-resistant cheater strain) are defined by the following set of functions:

$$G_W(t+1) = (\mu - c_{pg} + b \sum P_j) G_W(t) \quad (1)$$

$$G_P(t+1) = (\mu - c_{pg} - c_{tox} - c_{res} + b \sum P_j) G_P(t) \quad (2)$$

$$G_C(t+1) = (\mu + b \sum P_j) (1 - (\frac{\sum T_i}{\theta_T})^\kappa) G_C(t) \quad (3)$$

$$G_R(t+1) = (\mu - c_{pg} - c_{res} + b \sum P_j) G_R(t) \quad (4)$$

where c_{pg} , c_{tox} , c_{res} are the production costs per public good, toxin and the immunity mechanism, respectively. The term $\sum P_j$ stands for the number of public goods consumed by an individual and b for the benefit derived from this action. The term $\sum T_j$ represents the number of consumed toxins. Toxins decrease the overall growth rate and lead to death when they accumulate beyond the threshold value θ_T . The parameter θ_T can thus be understood as a measure of toxin potency. The negative effect toxins have on growth is further defined by the latency parameter κ . If $\kappa = 1.0$ then there is no latency and each toxin molecule has a linear additive negative effect on growth rate (Fig. S2). With $\kappa > 1.0$, susceptible cells can tolerate toxins at low uptake rates, while the negative effects of toxins on growth accelerate with increased toxin uptake. This accounts for the common phenomenon that bacteria possess non-specific resistant mechanisms (e.g. efflux pumps), thereby often tolerating low toxin concentrations (Fernández & Hancock, 2012, Nikaido & Pagès, 2012).

The public good producing strains W and P constitutively secrete one molecule per time step, whereas P additionally secretes toxins at the same rate. The diffusion of cells, public goods and toxins (described by the diffusion coefficients D_c , d_{pg} and d_{tox} , respectively) are modeled according to a Gaussian random walk, with a Gaussian random number generator based on the Box-Muller transform that converts uniformly distributed random numbers to normally distributed random numbers. Following diffusion, public goods and toxins are consumed whenever there is co-localization of molecules and cells.

Both public goods and toxins are represented by points on the landscape up to the precision of the computer (double precision) (Figs. 1 and S1). The molecules remain in the simulation until they are either consumed or decayed. The probability of decaying is determined by a durability value δ and an exponential decay function

$$P_{decay} = 1 - e^{-\omega \Delta^{T/\delta}} \quad (5)$$

where T is the current age of a molecule and $\omega = 0.1$ the steepness of the decay.

On our off-lattice landscape, individuals can physically overlap following cell growth and diffusion. To remove the overlaps, we implemented a procedure where overlapping cells are pulled slightly apart from each other in a random direction. The amount of pulling is determined by a maximum pulling distance, scaled by a uniformly distributed random number between 0 and 1. This procedure is repeated until the two cells are no longer overlapping. Once an overlap has been removed, the moved cells are checked again for potential new overlaps with other neighbouring cells. If the overlaps cannot be removed after a given number of trials and before the carrying capacity is reached, the current simulation is terminated, and a new one is started. In a final step, our stimulation involves a life/death control of each individual followed by the removal of dead cells that have passed the toxin threshold value θ_T . Fig. 2 depicts the order in which all the actions in our simulation were executed per time step (representing one second). We performed 50 independent replicates for each of the simulated parameter settings, and recorded the relative strain frequency at each time step.

We implemented two different simulation modes (Fig. 2). The first mode simulates competition assays, as they are typically conducted by microbiologists (Ross-Gillespie *et al.*, 2007). Two strains are mixed at equal frequency and allowed to compete during the exponential growth phase until the culture reaches carrying capacity. In this scenario, simulations are relatively short (max. 12,000 time steps) and terminate at the carrying capacity of $K = 1000$ cells. This type of simulation yields information on the selective advantage of different social strategies in growing populations, where public goods are most beneficial for nutrient scavenging (Griffin *et al.*, 2004, Diggle *et al.*, 2007, Kümmerli & Brown, 2010). The second mode simulates strain interactions across a longer time span (80,000 time steps) at an equilibrium frequency of approximately $K/2$. This mode resembles bacterial growth in a continuous (chemostat) culture, where population size is kept constant via a random cell removal mechanism. While the first mode is a useful approach to

understand the conditions under which a certain social behaviour is selected for or against, the second approach allows to estimate equilibrium frequencies of competing strains.

Explored Parameter Space

Table 1 provides an overview of all the parameters in our simulations and the value ranges explored. One key focus of our study is to understand how cell dispersal and molecule diffusion affect cooperation and policing. For cell dispersal D_c , we considered active but random bacterial motility, and varied this parameter from $0 \mu\text{m}^2/\text{s}$ (no dispersal) to $3.5 \mu\text{m}^2/\text{s}$ (high dispersal) in steps of 0.5. For molecule diffusion d_{pg} and d_{tox} , we implemented passive random diffusion processes and varied parameters from $1.0 \mu\text{m}^2/\text{s}$ (low diffusion) to $7.0 \mu\text{m}^2/\text{s}$ (high diffusion) in steps of one. Another important aim of our study is to explore how toxin properties affect policing. In addition to toxin diffusion, we varied: (a) toxin durability δ_{tox} from 50 (rapid decay) to 500 (intermediate decay) to 5000 (low decay) time steps; (b) toxin threshold θ_T from 600 (few molecules required to kill) to 1800 (many molecules required to kill) in steps of 400; and (c) toxin latency κ from 1.0 (low tolerance and immediate linear adverse effect) to 6.0 (high tolerance and delayed exponential adverse effect) in steps of 0.5.

Finally, the outcome of our simulations necessarily depends on the cost and benefit parameters. We implemented a basic growth μ to ensure that all cells can grow even if they do not produce public goods. This ensures that cells can only die because of toxins and not because they experience negative growth. A growth rate of $\mu = 1.0$ results in cell division occurring every 1,200 time steps, in the absence of molecule secretion. We fixed the cost and benefit of public good production to $c_{pg} = 0.001$ and $b = 0.01$. These values are based on our previous experience with the system (Dobay *et al.*, 2014) and ensure that public production generates a substantial net fitness benefit, thus significantly reducing cell division time (see also Fig. 3). Of key interest is how costly policing ($c_{tox} + c_{res}$) can be relative to c_{pg} in order to maintain a net benefit of cooperation. To address this question, we gradually varied the cost ratio of these two traits [$c_{pg}/(c_{tox} + c_{res})$] from 0.25 to 4.0. For all simulations, we assumed $c_{tox} = c_{res}$.

Strain Performance in Monoculture

In a first set of simulations, we assessed the performance of the strains W (wildtype cooperator), P (policing cooperator) and C (public-good cheater) in monoculture. These assays allow us to quantify (i) the net benefit of cooperation, and (ii) the acceptable boundary costs of policing to ensure a net benefit of cooperation. A net benefit of cooperation is given when the policing strain P grows better than strain C , which neither produces public goods nor toxins. Because we know that cell dispersal and secreted molecule diffusion influence the efficiency of cooperation (Dobay *et al.*, 2014), we performed simulations under three different diffusivity regimes, including high ($D_c = 4.0 \mu\text{m}^2/\text{s}$, $d_{pg} = d_{tox} = 7.0 \mu\text{m}^2/\text{s}$), medium ($D_c = 2.0 \mu\text{m}^2/\text{s}$, $d_{pg} = d_{tox} = 3.5 \mu\text{m}^2/\text{s}$) and low ($D_c = 0.1 \mu\text{m}^2/\text{s}$, $d_{pg} = d_{tox} = 1.0 \mu\text{m}^2/\text{s}$) diffusivity. We then compared the time needed by the three strains to reach carrying capacity.

Pairwise Strain Competition

We first simulated competitions between the strains *W* and *C* across the indicated range of dispersal and diffusion parameters to understand the conditions under which cooperation can be maintained in the absence of policing. Next, we competed *P* against *C*. In this scenario, *C* can still exploit the public good produced by *P*, but at the same time is harmed by the toxins secreted by this opponent. In a first set of simulations, we implemented intermediate default toxin properties ($d_{tox} = 4.0 \mu\text{m}^2/\text{s}$, $\delta_{tox} = 500 \text{ s}$, $\theta_T = 1000$, and $\kappa = 3.5$) to examine whether policing extends the range of conditions across which cooperation is favoured. In a second set of simulations, we then individually manipulated each of the four different toxin properties to identify the features required for toxin-mediated policing to be efficient.

Three-way Strain Interactions

To examine what happens when the genetic linkage between cooperation and policing breaks, we performed three-way competitions between strains *P*, *C*, and *R*. The latter strain no longer produces the toxin, but is resistant to it. Unlike in pairwise interactions, where we expect one strain to have a selective advantage during competition, non-transitive interactions might arise in three-way competitions, where strain frequencies can follow cyclical patterns (Kerr *et al.*, 2002). To allow for this possibility, we used the chemostat simulation mode to follow strain frequencies over extended periods of time (i.e. 80,000 time steps).

Results

Strain Performance in Monoculture

The growth of strain *C* (neither producing public goods nor toxins) was not affected by the diffusivity of the environment, and was solely determined by the basic growth rate μ , reaching carrying capacity after 12,000 time steps (Fig. 3). Strain *W* (producing public goods) grew significantly better than *C*, demonstrating the benefit of public goods cooperation. The growth of this strain was reduced in more diffusive environments, where the likelihood of public good sharing and consumption declines. The performance of strain *P* (producing toxins in addition to public goods) greatly varied both in response to the relative costs of policing and the diffusivity of the environment. In environments characterized by low diffusion, *P* always outperformed *C* even when the cost of toxin production was four times higher than the cost of public good production (Fig. 3). This was no longer the case in environments characterized by intermediate or high diffusivity, where *P* only grew better than *C* when toxins were cheaper than public goods (Fig. 3). If this condition was not met, then the high costs of policing combined with reduced public good consumption decreased cell growth to a point that prevented populations from reaching carrying capacity.

Interestingly, the relationship between the cost ratio $c_{pg}/(c_{tox} + c_{res})$ and the time needed to reach carrying capacity (τ_K) was best captured by the Monod equation (Monod, 1949), a hyperbolic equation initially used to explain the exponential growth rate as a function of nutrient concentration. Applied to our system, we found that the function

$$R(\tau_K) = R_K \frac{\tau_K}{R_\tau + \tau_K}, \quad (6)$$

provides a fair approximation to relate r to τ_K , where R_K represents the ratio limit for reaching carrying capacity and R_τ the time at which the carrying capacity is half the maximum (Fig. S3).

Low Cell Dispersal Favours Cooperation without Policing

When competing *W* against *C* without a policing mechanism in place, cooperation was only strongly favoured when cells did not disperse (Fig. 4A). In other words, if cooperator cells grew as microcolonies, physically separated from the cheaters, then efficient public good sharing among cooperators is promoted. We further found that neither cooperators nor cheaters had a clear selective advantage when cell and public good diffusion was low, but greater than zero (Fig. 4A). Under all other conditions, cheaters strongly dominated the community and pushed cooperators to very low frequencies.

Toxin-mediated Policing has Positive and Negative Effects for Cooperation

The introduction of a policing mechanism, which operates via the secretion of a toxin that selectively targets cheaters, had multiple dramatic effects on the competitive outcome between the cheater *C* and the cooperating policer *P* (Fig. 4B). Policing strongly increased selection for cooperation under conditions where cooperators could previously coexist with cheaters (compare Figs. 4A and 4B for combinations of low public good diffusion and cell dispersal). This finding demonstrates that policing can indeed extend the parameter space across which cooperation is favoured. Conversely, we found that policing also had negative effects and drastically accelerated selection against cooperation, especially under conditions where cheaters previously experienced only a moderate selective advantage (compare Figs. 4A and 4B for combinations of intermediate public good diffusion and cell dispersal). These two opposing effects led to a sharp transition between conditions that either completely favour *P* or *C*, leaving very few conditions where the two strains can potentially co-exist.

How Toxin Properties Affect Policing Efficiency

We found that higher toxin diffusion d_{tox} significantly increased selection for cooperation (Figs. 5A, 5E and 5B), especially under conditions of intermediate public good diffusion and cell dispersal. These results show that policing is particularly efficient when toxins are sent away to target more distant competitors whilst keeping the public good more local for preferential sharing among producers. Our simulations further revealed that high toxin durability δ_{tox} increases policing efficiency and thus selection for cooperation (Figs. 5C, 5E and 5D). These findings can be explained by the fact that more durable toxins are more likely to reach target cells. Next, we examined the role of toxin potency θ_T (i.e. the number of toxins needed to kill a target cell), and found that high potency is crucial for policing to promote cooperation (Figs. 5F, 5E and 5G). While this finding seems trivial at first sight, the dramatic effects we observed when decreasing toxin potency are remarkable. For instance, a reduction of toxin potency by 45 % (from $\theta_T = 1000$ to $\theta_T = 1800$) already completely

negated any benefit of policing, and even increased the parameter space across which cooperation was selected against. Finally, we explored the role of toxin latency κ for policing. Toxin latency is a measure of target cell tolerance: with low values of κ , the fitness of target cells is immediately affected in a linear way, whereas large values of κ mean that target cells can tolerate a certain level of toxins and negative effects only kick in later, but accelerate with higher toxin uptake rates (Fig. S2). We found that low κ values dramatically increased the efficiency of policing and selection for cooperation (Figs. 5H, 5E and 5I).

Policing goes Extinct when the Genetic Linkage between Traits Breaks

Next, we simulated the case where the genetic linkage between cooperation and policing breaks. Specifically, we studied the performance of strain *R*, which evolved directly from *P* by losing toxin production but keeping resistance, in competition with *P* and *C* across a range of intermediate environmental diffusivities. We found that the presence of *R* consistently drove *P* to extinction under all environmental conditions tested (Fig. 6). Our simulations, which kept populations at half the carrying capacity ($K/2 \sim 500$ cells) for an extended period of time allowed us to distinguish three distinct competition phases. The first phase comprises the time frame in which the community grows from three cells to $K/2$. During this phase, we observed cyclical fluctuations of strain frequencies with a general tendency for *C* to increase, *P* to decrease, and *R* to remain stable. The cyclical patterns observed here are reminiscent of the rock-paper-scissors dynamics described in previous studies on bacterial social interactions (Kerr *et al.*, 2002, Kelsic *et al.*, 2015, Inglis *et al.*, 2016), where strains chase each other in non-transitive interactions in circles with no overall winner. The second phase was characterized by a pronounced dip in *C* frequency accompanied by a strong increase in *R* frequency, and a moderate increase in *P* frequency in eight of the nine diffusion conditions (Fig. 6). This pattern is most likely explained by the accumulation of toxins in the environment, which efficiently suppressed *C*, but at the same time gave *R* leverage, as it could benefit from the effect of policing without paying the cost for it. During the third phase, we observed the extinction of *P*, the concomitant recovery of *C* followed by a decrease of *R* (Fig. 6). These patterns arise because as *P* decreases, toxin concentration declines allowing the recovery of *C*, which then efficiently exploits the public goods produced by *R*. In all cases, our simulations returned to a simple cooperator-cheater scenario, with the relative success of the two strategies being determined by the diffusivity of the environment (similar to the results in Fig. 4A).

Discussion

Understanding how policing, and the related concept of punishment, can repress competition and foster cooperation in social groups of higher organisms has attracted the attention of evolutionary biologists for decades (Frank, 1995, Clutton-Brock & Parker, 1995, Frank, 2003, West *et al.*, 2007b, Ratnieks & Wenseleers, 2008, Dreber *et al.*, 2008, Kümmerli, 2011, Raihani *et al.*, 2012, Cant *et al.*, 2013). Here, we explored whether policing could also be an effective way to enforce cooperation in groups of microbes, sharing beneficial public goods. In our models, policing is exerted via the secretion of a toxin that specifically targets cheater cells, which do not contribute to the pool of public goods. Our simulations reveal that policing is most conducive under conditions of intermediate cell dispersal and public

good diffusion, where it can extend the parameter space, under which cooperation is favoured. We further found that an effective policing mechanism entails a toxin that is: (i) cheaper to produce than the public good; (ii) more diffusible than the public good in order to effectively reach cheaters; (iii) durable; and (iv) potent in killing. However, our simulations also reveal two major downsides of policing. First, policing can accelerate the loss of cooperation under conditions where cheaters experience only a mild advantage in the absence of policing. This leads to a sharp state transition between conditions where policing either favours or disfavors cooperation. Second, policing is selected against if the genetic linkage between public good, toxin and toxin resistance breaks. If this occurs then toxin-resistant mutants that produce public goods, but no longer contribute to toxin production, derail policing, showing that microbial policing itself constitutes an exploitable public good (Inglis *et al.*, 2014).

Several studies have recently proposed toxin-based policing mechanisms to keep cheaters in check (Inglis *et al.*, 2014, Wang *et al.*, 2015, Majerczyk *et al.*, 2016, Evans *et al.*, 2018). While the idea of bacteria being able to punish cheating community members is exciting, our simulations reveal that specific ecological conditions are required for policing to be selectively favoured. When environmental diffusivity is very low then policing is not required because such conditions promote cooperation *per se*. The reason for this is that low public good diffusion and low bacterial dispersal lead to significant spatial segregation of strains within bacterial community, which promotes the local sharing of public goods among cooperators (Kümmerli *et al.*, 2009a, Mitri & Foster, 2013, Julou *et al.*, 2013, van Gestel *et al.*, 2014, Weigert & Kümmerli, 2017). Moreover, we found that policing is not favourable when environmental diffusivity is high, conditions that break any spatial association between cooperators and their public goods. Toxin-mediated policing can even be detrimental here because: (i) cheaters can freely exploit public goods; (ii) many toxins get lost due to the high diffusion, and thus never reach their targets; (iii) and the high level of cell mixing reduces the efficiency and selectivity of policing, as cooperators (albeit resistant) are hit by toxins as often as cheaters (Inglis *et al.*, 2011). Overall, intermediate levels of environmental diffusivity proved most beneficial for policing. For natural habitats, it is probably safe to assume that environmental diffusivity varies both temporally and spatially, which could quickly shift the selective balance for or against policing. It thus remains to be seen whether policing can evolve under fluctuating environmental conditions.

While we have implemented a generic toxin system in our model, it is important to evaluate which of the natural bacterial toxin systems could be most suitable for policing. Many toxin systems, such as colicins, are harboured on plasmids, where toxin and immunity genes are co-expressed as part of the same operon (Cascales *et al.*, 2007). On the one hand, the operon structure ensures strong genetic linkage between toxin and immunity, which would be good for the maintenance of policing. Conversely, plasmids are typically independent genetic units, such that regulatory linkage between plasmid-encoded toxin systems and chromosomally encoded cooperative traits might be rare. Other toxin systems, like pyocins produced by pseudomonads, are encoded on the chromosome and are also organised in operons (Michel-Briand & Baysse, 2002). Although suitable as potential policing systems, regulatory linkage between pyocins and quorum-sensing controlled cooperative traits has not been identified (Schuster *et al.*, 2003). Finally, bacteria can secrete toxic secondary

metabolites, such as cyanide and phenazines (Nadal Jimenez *et al.*, 2012) for which resistance occurs at least partly through the upregulation of efflux pumps (Sakhtah *et al.*, 2016). In *P. aeruginosa*, these two toxins are controlled by quorum sensing systems, and thus regulatorily linked to a number of cooperative traits (Nadal Jimenez *et al.*, 2012). While suitable and indeed identified as policing system by Wang *et al.* (2015), cyanide production and efflux pumps are encoded by different loci, which indicates that the regulatory linkage could be broken over evolutionary time scales (dos Santos *et al.*, 2018).

Another important point that has received little attention so far concerns the question whether the reported policing mechanisms have evolved as such or whether they represent by-products of regulatory linkage of traits, selected for other reasons than policing (West *et al.*, 2007c). For instance, in the case of *P. aeruginosa* it is well conceivable that cyanide primarily serves as a broad-spectrum toxin to target inter-specific competitors under high cell density (Bernier *et al.*, 2016). This might be the reason why the expression of cyanide is controlled by quorum sensing, and why it is regulatorily linked to other public good traits, such as protease production, whose expression is also controlled in a density-dependent manner (Darch *et al.*, 2012). Consequently, the observed cyanide-mediated policing exerted by wildtype strains on protease-deficient strains could be a mere by-product of this regulatory linkage. Alternatively, it is also possible that an initial co-incidental regulatory linkage between cyanide and protease later proved useful as a policing mechanism and evolved as such through cooperation (Foster, 2011). Clearly, further research is needed to uncover the evolutionary history of these putative policing mechanisms, and care must be taken to distinguish between mechanistic (proximate) and evolutionary (ultimate) explanation of observed behavioural patterns (West *et al.*, 2007c).

The potential policing mechanisms reported for microbes and the one implemented in our simulations differ in one important aspect from the policing systems described in animals and plants. Specifically, the difference is that the microbial policing mechanisms are genetically fixed (i.e. strains are either cooperating policers or cheaters), whereas the mechanisms reported in animals and plants reflect conditional strategy, which are deployed to sanction cheaters only when required (Frank, 1995, Clutton-Brock & Parker, 1995, Frank, 2003, Kiers *et al.*, 2003, West *et al.*, 2007b, Ratnieks & Wenseleers, 2008, Dreber *et al.*, 2008, Kümmerli, 2011, Raihani *et al.*, 2012). In the latter scenario, individuals can take decisions on whether to cheat or to cooperate, and whether to impose sanctions or not. In certain cases, it was found that the mere threat of policing was sufficient to coerce individuals to cooperate and prevent cheating in the first place (Wenseleers & Ratnieks, 2006, Cant *et al.*, 2013). Although conditional forms of cooperation and cheating via simple regulatory switches and feedback mechanisms have been reported for bacteria (Kümmerli *et al.*, 2009b, Cavaliere & Poyatos, 2013, Allen *et al.*, 2016, Pollak *et al.*, 2016, Rauch *et al.*, 2017), it remains to be seen whether the same mechanisms could also promote conditional policing. In the meantime, we argue that it seems fair to use the term ‘policing’ for the reported genetically fixed toxin-based systems, but also to keep in mind the difference between conditional and fixed social strategies.

In summary, our work contributes to the development of an evolutionary concept for policing in microbial systems. It shows how ecological factors, in particular the diffusivity of

the environment, interact with the properties of a toxin-mediated policing system, to define the parameter space in which policing can be favoured. It further demonstrates how realistic individual-based modelling, tracking both cells and their public goods over time and across space, can be used to deepen our understanding of social interactions in microbes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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To maintain the anonymity of the authors and their affiliations this section will be duly filled at the end of the review process. The authors declare no conflict of interest with this manuscript.

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Figure 1.

Point-in-time snapshots showing the spatial distribution of cooperating policers (in blue) and cheaters (in yellow) during competition. The point-in-time snapshots were taken during the early, intermediate and late simulation phase, depicting the growth of digital bacteria in their simulated environment. The density of public goods (in green) and toxins (in red) produced by the cooperating policers are indicated by the intensity level of their respective color. The specific parameters values for this example are: $D_c = 0.0 \mu\text{m}^2/\text{s}$, $d_{pg} = 1.0 \mu\text{m}^2/\text{s}$, $d_{tox} = 2.0 \mu\text{m}^2/\text{s}$, $\delta_{tox} = 500 \text{ s}$, $\theta_T = 1000$ and $\kappa = 3.5$.

Figure 2.

Flow diagram of the computer simulation showing the order of each procedure called during a single time step (i.e. one second). Our platform allows for two different types of culturing conditions: batch-culture growth, where simulations stop when populations reach the carrying capacity $K = 1000$ (solid lines); and continuous growth in a chemostat, where simulations stop after a given time period (dashed lines). To guarantee continuous growth, the chemostat cycle comprised a random cell removal mechanism, such that population size was kept constant at $K/2$. Each box describes a key element in a cell's life cycle, affecting its

fitness: i.e. cells grew according their fitness equations (see main text) and divided when reaching the threshold radius of $1\ \mu\text{m}$ (two times the initial radius). Since cells are free to move on a continuous landscape, they can overlap following diffusion. To remove cell overlap, we used a procedure based on the physics of hard-core interactions used in molecular dynamics and cell elasticity.

Figure 3.

Growth in monoculture and the relative cost of policing. We examined how large the cost of policing ($c_{tox} + c_{res}$) can be relative to the cost of public good production (c_{pg}) to guarantee a net benefit of cooperation. To address this question, we compared the growth of the policing strain (producing toxins and public goods, in blue) with the growth of the cooperator strain (producing only public goods, in red) and the cheater strain (producing neither toxins nor public goods, in yellow) in monoculture for a range of cost ratios, under three different diffusion regimes. The yellow bar indicates that the time needed by the cheater strain to reach carrying capacity was not affected by the diffusivity of the environment. The red bars show that the time needed by the cooperator strain to reach carrying capacity increased with higher diffusivity of the environment (plain line: low diffusivity, $D_c = 0.0 \mu\text{m}^2/\text{s}$, $d_{pg} = d_{tox} = 1.0 \mu\text{m}^2/\text{s}$; dashed line: intermediate diffusivity, $D_c = 2.0 \mu\text{m}^2/\text{s}$, $d_{pg} = d_{tox} = 3.5 \mu\text{m}^2/\text{s}$; dotted line: high diffusivity, $D_c = 4.0 \mu\text{m}^2/\text{s}$, $d_{pg} = d_{tox} = 7.0 \mu\text{m}^2/\text{s}$). For policing to evolve, the time needed by the policer strain to reach carrying capacity must be within these boundaries (low diffusivity: blue triangles; intermediate diffusivity: diamonds, high diffusivity: dots). Values represent averages of 50 independent simulations. Standard errors of the mean are smaller than the size of the markers and therefore not shown. We set $c_{pg} = 0.001$, while varying $c_{tox} + c_{res}$ from 0.00025 to 0.004. The black encircled values

correspond to $c_{pg}/(c_{tox} + c_{res}) = 1.5$, the relative policing cost used for all subsequent simulations.

Figure 4.

Competition between cheaters and cooperators across a range of cell dispersal D_c and public good diffusion d_{pg} coefficients. Heat maps depict the frequency of the cooperator strain after the community reached stationary phase. (A) Outcome of competitions between the public-good-producing cooperator W and the cheater C in the absence of a policing mechanism. (B) Outcome of competitions between the policing cooperator P and the cheater C . D_c varied from 0.0 to $3.5 \mu\text{m}^2/\text{s}$, whereas d_{pg} varied from $1.0 - 7.0 \mu\text{m}^2/\text{s}$. The other parameters were set to intermediate values: $d_{tox} = 4.0 \mu\text{m}^2/\text{s}$, $\delta_{tox} = 500 \text{ s}$, $\theta_T = 1000$, $\kappa = 3.5$.

Figure 5.

Heat maps exploring the effect of different toxin properties on the success of cooperation. We varied toxin diffusion d_{tox} , toxin durability δ_{tox} , toxin potency θ_T and toxin latency κ across a range of values (see Table 1) and show here heat maps of the frequency of the cooperator strain after the community reached carrying capacity for the lowest, highest and intermediate parameter values. At the center of the figure we placed a reference heat map (E), where all the parameters were set to intermediate values ($d_{tox} = 4.0 \mu\text{m}^2/\text{s}$, $\delta_{tox} = 500 \text{ s}$, $\theta_T = 1000$, $\kappa = 3.5$). We then varied toxin diffusion from 1.0 to $7.0 \mu\text{m}^2/\text{s}$ (A, E and B),

toxin durability from 50 to 5000 (C, E and D), toxin potency from 600 to 1800, (F, E and G) and toxin latency κ from 2.0 to 6.0 (H, E and I). We only varied a single parameter at the time, and kept all others at their intermediate default value used for (E).

Figure 6.

Policing is selected against when the genetic linkage between public good and toxin production breaks. Panels show simulated three-way interactions between cheaters C (yellow lines), cooperating policers P (blue lines), and toxin-resistant non-policing cooperators R (red lines) across a range of public good and cell dispersal values. We assume that R can directly evolve from the policing strain P when the genetic linkage between public good and toxin production breaks. If this happens, our simulations reveal that policers are always selected against. Trajectories show strain frequency fluctuations in continuous cultures over 80,000 time steps. Values depict means of 50 independent replicates with the standard error of the mean (transparent areas). For all simulations, we kept toxin properties constant: $d_{tox} = 7.0 \mu\text{m}^2/\text{s}$, $\delta_{tox} = 500 \text{ s}$, $\theta_T = 1000$, $\kappa = 3.5$.

Table 1

Item	Parameter	Description	Range
Cell	r	radius	0.5 - 1 μm
	D_c	dispersal	0 - 3.5 $\mu\text{m}^2\text{s}^{-1}$
	μ	growth rate	1.0
Public good	p_{pg}	production rate	1.0 s^{-1}
	c_{pg}	production cost	0.001
	b	benefit	0.01
	d_{pg}	diffusion	1.0 - 7.0 $\mu\text{m}^2\text{s}^{-1}$
	δ_{pg}	durability	500 s
Toxin	p_{tox}	production rate	1.0 s^{-1}
	$c_{tox} + c_{res}$	production & immunity cost	0.00025 - 0.004 [*]
	c_{tox}	production cost	0.0003 [†]
	c_{res}	immunity cost	0.0003 [†]
	d_{tox}	diffusion	1.0 - 7.0 $\mu\text{m}^2\text{s}^{-1}$
	δ_{tox}	durability	50 - 5000 s
	θ_T	potency	600 - 1800
	κ	latency	1 - 6

^{*} varied in the simulations shown in Fig. 3.

[†] based on the simulations shown in Fig. 3, c_{tox} and c_{res} were fixed to these values for all subsequent analyses.