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ORIGINAL PAPER

Modeling bacterial quorum sensing in open and closed environments: potential discrepancies between agar plate and culture flask experiments

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Abstract Quorum sensing (QS) is a process of bacterial communication and cooperation mediated by the release of jointly exploited signals and "public goods" into the environment. There are conflicting reports on the behavior of mutants deficient in the release of these materials. Namely, mutants that appear perfectly viable and capable of outgrowing wild type cells in a closed model system such as a culture flask, may not be viable or invasive on open surfaces such as agar plates. Here we show via agent-based computational simulations that this apparent discrepancy is due to the difference between open and closed systems. We suggest that the experimental difference is due to the fact that wild type cells can easily saturate a well-mixed culture flask with signals and public goods so QS will be not necessary after a certain time point. As a consequence, QS-deficient mutants can continue to grow even after the wild type population has vanished. This phenomenon is not likely to occur in open environments including open surfaces and agar plate models. In other words, even if QS is required for survival, QS deficient mutants may grow faster initially in short term laboratory experiments or computer simulations, while only WT cells appear stable over longer time scales, especially when adaptation to changing environments is important.

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Introduction

Quorum sensing (QS) is a cell–cell communication process in which bacteria release and detect molecular signals called autoinducers, which enables them to monitor cell population density and make a variety of coordinated responses [1].[1] Acyl homoserine lactones (AHLs), the major class of autoinducer signals used by Gram-negative bacteria, consist of a conserved homoserine lactone ring coupled to an acyl side chain, which may vary from 3 to 18 carbons in length. All AHLs are believed to diffuse freely across the cell envelope; however, efflux pumps may actively export some longer chain AHLs [2]. In a typical AHL-QS circuit, the AHL signals are synthesized by a LuxI-type protein. At a critical concentration, the AHL binds to a LuxR-type protein, and the LuxR-AHL complex acts on target genes that affect a variety of cellular processes [3] including the production of exoenzymes and exopolysaccharides. As these QS-regulated factors are released into the environment and are accessible to other cells, they are often referred to as "public goods", i.e., openly accessible means of intercellular cooperation. For instance, cells of the ubiquitous opportunistic pathogen *Pseudomonas aeruginosa* have two AHL-QS systems (see, e.g., [4, 5]). In

[1] Definition of terms used in this paper: *QS* Quorum sensing; *QS system*: a QS system of *Psuedomanas aeruginosa* consists of two fundamental genes, abbreviated here as *R* and *I; rhl* the QS system of *P. aeruginosa* that controls rhamnolipid production. It consists of the genes *rhlR* and *rhlI; las* the QS system of *P. aeruginosa* that controls elastase production. It consists of the genes *lasR* and *lasI*; *SN* a non-communicating mutant with the *I* gene (either *lasI*, *rhlI* or both) deleted. These mutants do not produce the signal, but react to it. *SB* a non-cooperating mutant with the *R* gene (either *lasR*, *rhlR* or both) deleted. These mutants produce a very low level of signal, but do not respond to it.

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the *las* system, the LasI protein produces an *N*-(3-oxo-dodecanoyl)-homoserine lactone signal that binds to the LasR regulator protein and activates the production and release of an elastase enzyme that will digest proteinaceous nutrients in the environment into amino acids that the bacteria can utilize directly. In the *rhl* system, the RhlI protein produces *N*-(butanoyl)-homoserine lactone and activates the RhlR protein, which will trigger genes involved in rhamnolipid production. The rhamnolipids released facilitate the movement of swarming cells. Importantly, the regulatory circuitry that contains *luxI* and *luxR* genes ensures a rapid amplification of signal production and, as a result, the populations can switch states swiftly in a coordinated manner if required by the environmental conditions. The two AHL systems of *P. aeruginosa* are interlinked, which can give rise to complex cellular responses. For instance, mutants containing a single deletion in the *las* system affect several pathways. This is all the more remarkable since some Gram-negative bacteria use the same type of AHL signals. Sharing of signals and other public goods can then lead to a coordinated behavior of multispecies consortia in which different species communicate and cooperate.

The role of the *luxI* and *luxR* genes can be studied conveniently using deletion mutants [6]. Cells in which the *luxI* gene is deleted will not produce the signal, so they are termed "signal negative" or "SN". Even though these cells cannot produce the signal above a very low, baseline level, they can respond to signal molecules produced by other bacteria, for instance by producing public goods. In other words, SN cells cannot communicate but can cooperate. Cells in which the *luxR* gene is deleted cannot respond to the signal so they are termed "signal blind" or "SB". SB cells are not able to upregulate their signal production, nor do they produce public goods. In other terms SB cells neither communicate, nor cooperate.

The behavior of AHL QS mutants of *Pseudomonas aeruginosa* has been studied both in laboratory models and with computational models. Diggle et al. [6] studied the experimental behavior of Δ*lasI* (SN^L) and Δ*lasR* (SB^L) deletion mutants in shake cultures grown in defined medium. In this medium, SN^L and SB^L mutants grew slower than the wild type (WT). Adding exogenous signal molecules to the system restored the growth of SN^L to WT levels, but not to those of SB^L. If, however, the mutants were grown in pairwise competitions with the WT strain, both were able outgrow WT cells. This phenomenon was ascribed to the lower metabolic costs of deletion mutants; however, one could also argue that the active *rhl* system may also have contributed to the growth of the mutants. For this reason, Venturi and coworkers [7] constructed Δlas ΔrhlI (SN^{LR}) and *lta*las ΔrhlR (SB^{LR}) double deletion mutants, and studied their behavior on swarming agar plates. On these plates WT *P. aeruginosa* cells formed branched colonies typical of swarming cells, but neither SN^{LR} nor SB^{LR} cells could swarm alone. Non-swarming colonies remained at the starting position without any visible sign of growth. In pairwise competitions, $WT+SN^{LR}$ cells formed branched colonies only slightly smaller than those of pure WT populations. $WT+SB^{LR}$ cells, however, could not swarm, but formed a colony slightly bigger than the starting population, as if the population starting to grow would suddenly collapse. The behavior of SN and SB mutants was studied by computational models endowed with a single QS system and it was found that the fundamental findings, i.e., the co-swarming of WT+SN cells, and the collapse of WT+SB populations after an initial growth phase could be reproduced qualitatively by agent populations whose growth and mobility was controlled by a single QS regulatory circle. This finding suggested that QS alone can explain the behavior of mutant populations; however, the question remains why SB^L mutants can outgrow WT cells in pairwise competitions in liquid culture while $WT+SB^{LR}$ populations collapse on agar plates. One of the immediate answers could be the difference between SB^L and SB^{LR} mutants, i.e., SB^L mutants may grow better because of their active *rhl* system, i.e., there may be a difference between single and double knockout mutants. This is not the case, however, since the behavior of the single and double mutants (both SN and SB) was indistinguishable when tested on agar plates [7]. This identical behavior led us to speculate that the difference may be due to some aspect of the test system, probably the open or closed nature of the experimental system. The question whether or not SN and SB mutations can invade WT colonies is interesting both in theory and for medical applications. A population is considered evolutionarily stable if deleterious mutations cannot invade it. In shaken cultures, both SN and SB mutants could be considered invasive as they outgrow WT cells. Consequently, WT cells could be considered as evolutionarily unstable. Not so, however, on agar plates where the collapse of WT+SB populations leads to the local extinction of both partners. The locality of the collapse is important since it ensures that other parts of the WT colony can continue to grow undisturbed. In this sense, WT cells can be considered as evolutionarily stable. QS mutations of *P. aeruginosa* are also important from a medical standpoint as stable SN^L and SB^L colonies were found in the lungs of cystic fibrosis patients and these mutants are viable in vivo [8].

Here, we test the hypothesis that the different and seemingly contradictory results reported previously are due to the differences between closed and open systems. Namely, well stirred, shaken cultures can be quite rightly considered as closed systems where each cell of the populations can be in contact and can influence the behavior of any other cell in the population. Agar plates, on the other hand, mimic open surfaces inasmuch as the medium is not stirred so that cells interact only with their close neighbors. Even though the natural environments of bacteria can be considered only

approximately closed or open, we have to note that QS signaling is profoundly affected by the closeness/openness of the environment. For instance, a single bacterial cell will turn on QS if placed in a very small volume, and similar conditions can exist when bacterial cells reach the microcapillaries of plants. Or, the environment of free-floating marine bacteria is near to an ideally open system, but as the cells adhere to a particle of organic waste they enter a more closed environment. Here they can start growing and use QS to activate molecular mechanisms such as enzyme production that will allow them to digest the particle. In other terms, QS bacteria are adapted to live in a variety of environments that constitute various degrees of transitions between ideally closed and ideally open systems.

In this paper we use in silico modeling to study the behavior of WT *P. aeruginosa* as well as its SN and SB mutants in open and closed environments and show that the previous, seemingly contradictory results are not artifacts but are in fact due to the differences between open and closed experimental systems.

Results and discussion

We carried out two types of experiments: competition and invasion. In competition experiments, the participating species were present in equal numbers. In invasion experiments, a small number of mutant cells were added to a large population of wild type agents.

In both cases we used two types of environment, the so called open and closed models. The concept of the two models is shown in Fig. 1.

The open model in our case means a longitudinal surface where bacterial agents are placed at the bottom and move upwards during the simulation (Fig. 1b). The surface is divided into squares in which the concentration of the solutes (signal, factor and nutrient) levels are the same. There is finite amount of nutrient on the surface, which decreases as bacteria consume it. As a result, nutrient will diffuse to the square from areas with higher nutrient concentration. In a similar way, the signal and public goods produced by the cell also spread via diffusion.

A closed system is simpler, there is only one spatial unit ('square'—i.e., toroid surface) (Fig. 1a), so the concentration level of nutrient, signal and public goods is constant throughout the whole system. We introduced an infinite amount of nutrient otherwise all simulations ended with the bacteria (WT or mutant) perishing by starvation. In order to obtain realistic, sigmoidal growth curves, we maximized the number of bacteria in a way that the agents consumed less energy as they approached the population limit—a standard approach in many areas of biological modeling [9]. All calculations were carried out with explicit representation of cells, diffusible signals and public goods as described in [10–12] and shown in Fig. 1.

Competition experiments

In competition experiments our goal was to examine the behavior of competing species where the participating

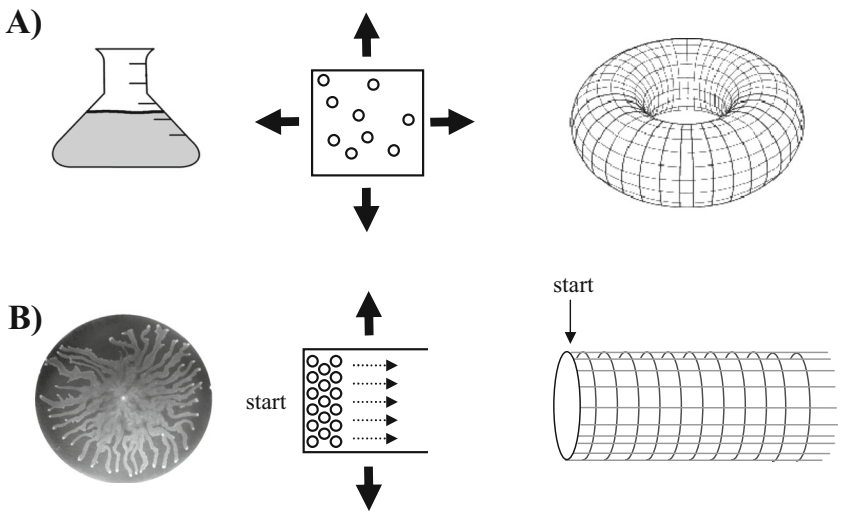

Fig. 1a,b Modelling closed and open systems in 2D. **a** A closed system (culture flask) is represented as a *square* in which *arrows* indicate periodic boundary conditions on all sides (*left*), which corresponds to a toroidal surface (*right*). **b** The open system (agar plate) has periodic boundary conditions on two sides only, indicated by *black arrows* (*left*), which corresponds to a cylindrical surface open on one end (*right*). *Circles* indicate cell agents. The stating population is distributed randomly all over the closed system. In the open system the cell agents are positioned randomly in the vicinity of the "start", and during the modeling experiment the (growing) community spontaneously proceeds as indicated by the *dotted arrows*

populations (WT and a mutant) were present in equal numbers. We examined this in four cases: WT + SN and WT + SB species, on open and closed surface (Fig. 2). SN mutants form a stable community with the WT species, both in the open (top left) and in the closed system (bottom left) and, in both cases, the mutants grow faster than WT bacteria. On the other hand, the SB mutant can outgrow the WT only in the closed system (bottom right). In the open system the mixed population collapses (top right). Note that our computational models have a single QS system so we use the simple notation SB and SN for the mutants, without the upper indices.

Results of the computational model are similar to those obtained by Venturi et al. [7] and Diggle et al. [6]. As already mentioned, Venturi and coworkers saw swarming populations in WT plus SN competitions, but collapsing, not swarming, populations when adding SB mutants to WT agents, like in our open model. In the world of Diggle and associates both mutants were able to outgrow WT cells in pairwise competitions similarly as agents behaved in our closed model. These results suggest that the difference in the two experiments can be explained by the difference in the environments, rather that with the difference in the mutant species used in the two studies (single knockout mutants in Diggle et al. [6] and double knockout mutants in Venturi et al. [7]).

The difference between the two experimental systems can be further characterized by considering the nutrient supply and the response of bacterial populations. In open environments, a population proceeds constantly towards pristine areas of the agar plate; however, the local nutrient supply is exhaustible and must be competed for. This leads to the collapse of QS signaling in some cases, and there will be no growth even though nutrients are still available. In closed, well-mixed, submerged cultures, experimenters usually add a large amount of nutrients to the culture, which is not exhausted for the duration of the experiment, which is typically less than 48 h, and a growth inhibition effect will stop the growth of populations before all nutrients are exhausted. But can collapse be reached in a submerged culture? We believe not. Specifically, as long as there are nutrients in the system, both competing populations will grow, and since the nutrients are distributed

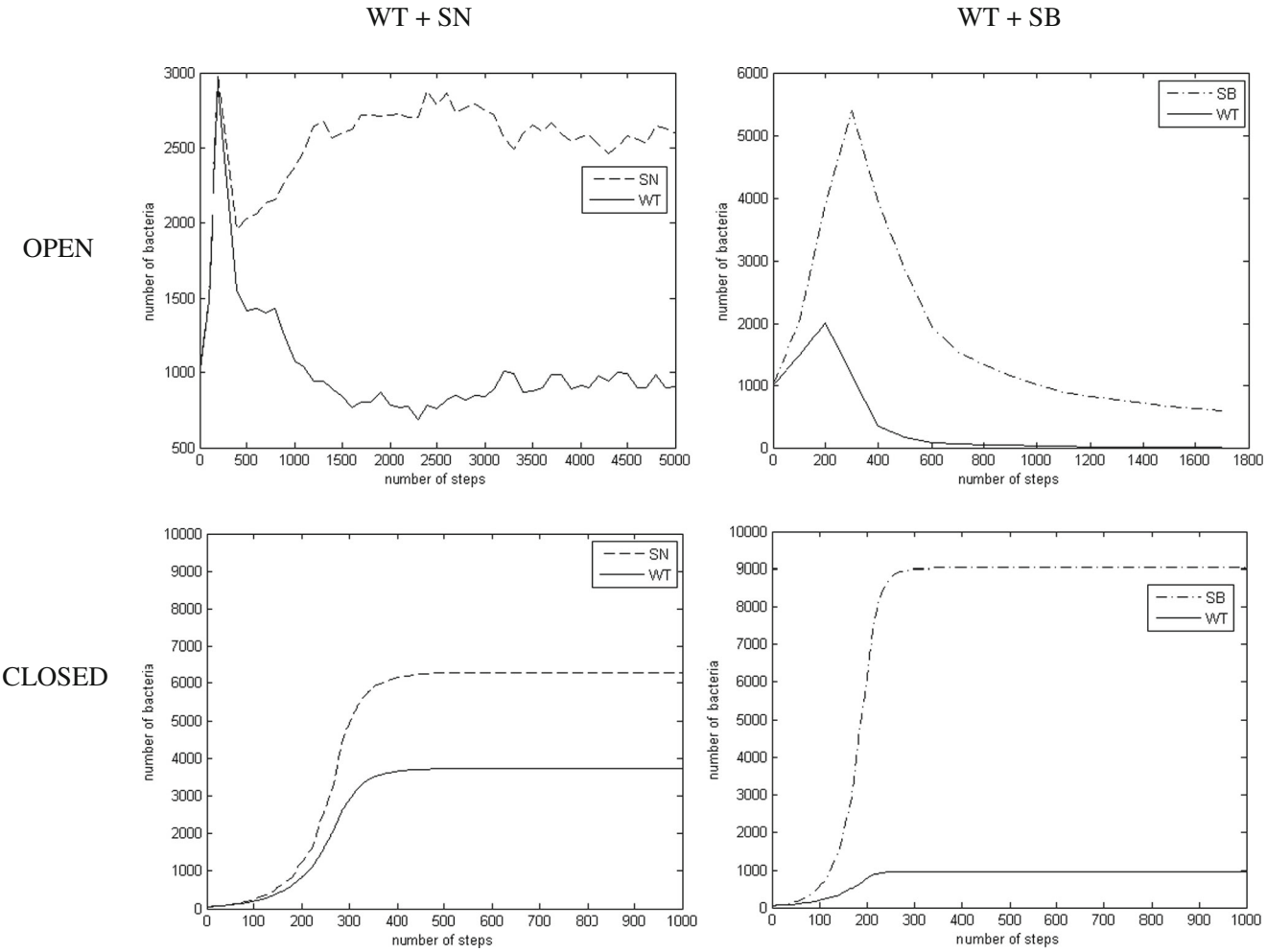

Fig. 2 Competition of WT and mutant bacterial models in open (*top*) and closed (*bottom*) systems. The experiments were set up with an equal number of WT (*solid line*) and mutant (*dashed line* SN, *dash-dotted lines* SB) cells and the population size was plotted as a function of time

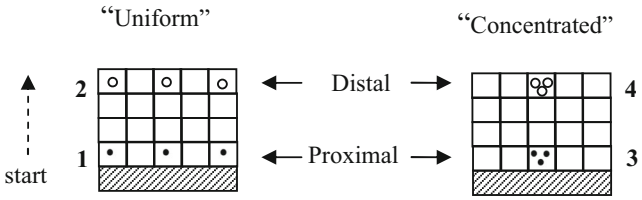

Fig. 3 Modeling position and density dependence in an open system. **a** Uniform distribution means equidistant positioning of agents. **b** Concentrated distribution means all agents are placed at a minimal allowed distance from each other. Proximal positioning means placing the agents close to the boundary of the system while distal means placing it further apart. Note that proximal/distal positioning is only possible in our open system as the closed system has no fixed boundaries

evenly, growth will be detected throughout the entire system. Moreover, it is a well known experimental fact that spent media contain large amounts of signals and public goods. We can simplify this situation by saying that a closed flask is easily saturated with signals and public goods; thus, after a certain time QS will not be necessary for survival. This is not the case in realistic, open systems. We can summarize the results by stating that agar plate experiments show the phenomenon of collapse characteristic of natural environments, while mutants that are not viable in nature can show up as winners in stirred flask cultures.

Invasion experiments

Invasion experiments are meant to determine whether or not a given mutant is able to spread in a WT population. Our goal was to determine the "invadability" of a mutant in open and closed systems. Exploratory experiments showed that the position and the number (local density) of the invading mutant has a remarkable effect on the success of invasion in open systems. When the invading mutants were positioned far from each other and near the system boundary (position "1" in Fig. 3), the success rate of the invasion was usually lower than in the opposite case (position "4" in Fig. 3). Typical results are shown in Table 1. It was also noted that the models behave in a stochastic way, i.e., the same number of mutants positioned in the same way invaded the community in only a fraction of the experiments. Large number of experiments were carried out in which the density of the invading mutants was systematically varied (Table 2). We found that both the non-communicating and the non-cooperating mutants can invade the WT community by growing initially faster than the wild type.

Our simulations thus show that both SN and SB mutant species can invade the wild type cultures in a closed environment, regardless of their initial density (Table 2). Simulations in open cultures show a stochastic behavior, and SN and SB can invade the WT population; however, the outcome is either collapse (SB mutants) or a stable mixed community (SN mutants).

We mention that both the theoretical and the experimental aspects of QS are the subject of intensive studies (for an extensive review see [13]). The most frequent goal is to discuss the long term evolutionary stability of cooperation, of which bacterial QS is often considered to be a good model. Our general approach is different: (1) we study the role of QS in short-term community formation; (2) We use agent-based, i.e., particle-like models better known in physicochemistry and physics. So we cannot make strong claims about the evolutionary fate of mutants, we can only suggest that mutants that die out in short times are probably not stable on the evolutionary time scales either. More importantly, we believe that QS is meant to facilitate bacterial survival in changing

Table 1 Typical results for the position and density dependence of invasion experiments. Fifty non-communicating SN mutants + 1,950 wild type agents placed in different ways shown in Fig. 3. The numbers in parenthesis refer to the positions indicated in Fig. 3

	Proximal	Distal
Uniform	"–"(1)	"+"(2)
Concentrated	"+"(3)[a]	"+"(4)

[a] Stochastic coexistence occurred in 35 % of cases (in the other three cases the observed outcome could be obtained in 100 % of the simulations)

Table 2 Invadability in open and closed models. "+" means that mutant species could grow, resulting a coexistence for SN, and a collapse for SB mutants. Values in parenthesis represent the % of cases that the "+" outcome was observed in 20 repetitions

Type of invasion	Response in the closed space model	Response in the open space model
Non-communicating mutant SN		
1 SN : 1,999 WT	"+" (100 %)	"+" (0–65 % coexistence)[a]
50 SN : 1,950 WT	"+" (100 %)	"+" (0–100 % coexistence)
1,000 SN : 1,000 WT	"+" (100 %)	"+" (100 %)
Non-cooperating mutant SB		
1 SB : 1,999 WT	"+" (100 %)	"+" (0–55 % collapse)
50 SB : 1,950 WT	"+" (100 %)	"+" (0–100 % collapse)
1,000 SB : 1,000 WT	"+" (100 %)	"+" (100 % collapse)

[a] Stochastic, position and density dependent response

environments, so it may become lost only in environments where wide adaptation is no longer important. A shake culture flask, saturated with signals and public goods is such an environment.

Conclusions

The behavior of QS bacteria is often characterized in laboratory experiments, especially in closed systems such as well stirred submerged cultures. In silico modeling experiments presented here show that QS deficient mutants that are not viable in an open environment may appear viable and capable of invading wild type colonies in closed systems. We suggest that this difference is due to the well known fact that wild type cells can easily saturate a culture flask with signals and public goods so that QS deficient mutants can continue to grow even after the wild type population has vanished, since they do not need an intact QS system—a condition rarely met in natural environments. As QS bacteria often shuttle between different environments, differential behavior with respect to open and closed systems may need to be considered when describing the viability of mutants. Importantly, while QS mutants grow initially faster in short term laboratory experiments or computer simulations, only WT cells are deemed to be stable over longer time scales, where adaptation to fluctuating environments is important.

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