*FEMS Microbiology Ecology*, 2023, **99**, 1–10
DOI: 10.1093/femsec/fiad128
Advance access publication date: 12 October 2023
Minireview

# To cheat or not to cheat: cheatable and non-cheatable virulence factors in *Pseudomonas aeruginosa*

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**Editor:** [Marcus Horn]

## Abstract

Important bacterial pathogens such as *Pseudomonas aeruginosa* produce several exoproducts such as siderophores, degradative enzymes, biosurfactants, and exopolysaccharides that are used extracellularly, benefiting all members of the population, hence being public goods. Since the production of public goods is a cooperative trait, it is in principle susceptible to cheating by individuals in the population who do not invest in their production, but use their benefits, hence increasing their fitness at the expense of the cooperators' fitness. Among the most studied virulence factors susceptible to cheating are siderophores and exoproteases, with several studies *in vitro* and some in animal infection models. In addition to these two well-known examples, cheating with other virulence factors such as exopolysaccharides, biosurfactants, eDNA production, secretion systems, and biofilm formation has also been studied. In this review, we discuss the evidence of the susceptibility of each of those virulence factors to cheating, as well as the mechanisms that counteract this behavior and the possible consequences for bacterial virulence.

**Keywords:** biofilms; biosurfactants; exoprotease; pyocyanin; secretion systems; siderophores; social cheating

## Introduction

In principle, all cooperative behaviors are susceptible to cheating, and the production of public goods by microbes is no exception (Smith and Schuster 2019). Interestingly, many examples of public goods involving bacteria are virulence factors, increasing bacterial virulence and hence favoring infections, nevertheless, since they are social traits, they are in principle also susceptible to cheating by non-producer individuals. For cheating to take place, the expression of the cheatable trait must be costly, in terms of the energy and resources required to produce it, and the benefit derived from the production of such a factor should not be restricted to the individuals who produce it but should have the potential of benefitting other members of the population. Thus, when cheating occurs, the fitness of the cheater increases at the expense of the fitness of the cooperator (García-Contreras and Loarca 2021). Cheaters are usually generated by mutations that decrease or preclude the production of a cooperative trait, usually in genes encoding master regulators such as LasR that activate the expression of several quorum sensing (QS) controlled virulence factors such as exoproteases, phenazines, rhamnolipids, etc., or genes encoding sigma factors such as PvdS that control the expression of the genes involved in the biosynthesis of pyoverdine, the main siderophore in *Pseudomonas aeruginosa* (Sandoz et al. 2007, Wilder et al. 2011, Dandekar et al. 2012, Granato and Kümmerli 2017, Loarca et al. 2019, Tostado-Islas et al. 2021). However, another way to cheat at least temporarily without necessarily being a mutant could be by the transitory lack of expression of the cooperative trait; however, that strategy has been less explored so far. It is well established that during chronic infections often *lasR* and *pvdS* mutants are found, hence suggesting a cheating behavior *in vivo*, although other explanations for their selection have been proposed (De Vos et al. 2001, Hoffman et al. 2009, Meijerink et al. 2010, Jiricny et al. 2014, Hennemannid et al. 2021).

Regardless of the detrimental effects of cheating for bacterial populations, non-cooperative individuals for some traits may cooperate in other ways, either in situations of regular growth or under stressful conditions; moreover, since sometimes the production of extracellular metabolites exceeds the optimal (their cost in terms of energy would exceed the benefit of their utilization), a positive effect of the cheaters would be to decrease the overall production of such metabolites, hence decreasing the resources used in their synthesis and export, leaving more energy available for metabolic processes linked to growth and biomass production. Moreover, as cheating decreases, the overall production of virulence factors that are sometimes immunogenic is thought to contribute to a transition from acute to chronic infections and it has been demonstrated that several social traits including QS, biofilm, and siderophore production decrease in chronic *P. aeruginosa* infections as a function of time (García-Contreras and Loarca 2021).

Hence, in principle, the consequences of virulence factor cheating for pathogenic bacteria could be complex, since they may decrease the overall virulence of infections, by decreasing the

**Received** 23 July 2023; **revised** 30 August 2023; **accepted** 11 October 2023
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amount of virulence factors produced and the fitness of the producers, increasing the fitness of the cheaters that may be less harmful to the host. On the other hand, cheating may contribute to the attenuation of immune responses by decreasing the expression of immunogenic factors and hence may contribute to the creation of chronic infections.

## Virulence factors

### Exoproteases and siderophores

*Pseudomonas aeruginosa* is an opportunistic pathogenic bacterium, responsible for around 10% of nosocomial infections worldwide, which very frequently presents resistance to multiple antibiotics and is therefore very difficult to eradicate (Poole 2011), this bacterium has a plethora of virulence factors that it uses to establish its infections, including various exoproteases such as LasA and LasB elastases, AprA collagenase, or protease IV, whose expression is positively regulated by the QS system. Moreover, it synthesizes the siderophores pyoverdine and pyochelin, which are produced preferentially when the bacterium grows in low iron concentrations (Castillo-Juárez et al. 2015). A common characteristic of siderophores and exoproteases is that they are molecules that are released into the environment to fulfill their functions: hydrolyze proteins, release usable peptides and amino acids, and administer iron to bacteria, respectively; therefore, the individuals that produce them have no control over their use and this is why they are considered public goods, the production of which is a cooperative phenomenon (Smith and Schuster 2019). Cooperative behaviors are exploitable by opportunistic individuals who do not cooperate but enjoy the benefits provided by public goods; this was demonstrated in bacteria in 2007, using *P. aeruginosa* and exoprotease production as a model. In two independent studies, it was shown that exoprotease production is metabolically expensive but essential for bacteria to grow properly in a minimal medium, with protein as the only carbon source, and that in this medium, mutants with a dysfunctional QS system due to mutations in *lasR*, the gene that encodes the main receptor for quorum signals, do not produce exoprotease and can only grow in the presence of producer individuals, behaving as opportunists (social cheaters). In addition, these mutants appear spontaneously and are selected in serial cultures in this medium where, if they accumulate at high frequencies (80%–90%), they cause the culture to no longer grow, that is to say, a population collapse is reached, which in ecology is known as the "tragedy of the commons"(Diggle et al. 2007, Sandoz et al. 2007). Similarly, in iron-limited culture media, non-producing mutants of the main siderophore (pyoverdine) arise, mainly due to mutations in the gene encoding the sigma factor PvdS, which controls the transcription of pyoverdine biosynthesis pathway genes; the accumulation of these mutants can likewise lead to population collapses (Griffin et al. 2004). However, most often mutant populations establish equilibrium with producer individuals, stabilizing at mutant frequencies of around 60%–70% (Dandekar et al. 2012). It is important to mention that these types of mutants are not only observed in the laboratory but also appear frequently in chronic infections (De Vos et al. 2001, Jiricny et al. 2014), and it has even been shown that in animal infections the presence of these mutants attenuates bacterial virulence; experimental therapies have been designed that involve their use, and use in patients is being considered (Rumbaugh et al. 2009). In recent years, various working groups, including ours, have investigated the role of different aspects that influence population dynamics, equilibrium, and collapses in cultures that contain mixtures of cooperative individuals and mutants, demonstrating that factors such as the segregation of cooperators in structured environments favor the preferential use of public goods by them, thus restricting the benefit to opportunistic mutants. In addition, *lasR* mutants are more sensitive to oxidative stress because they express lower levels of enzymes such as catalase and superoxide dismutase; therefore, the addition of hydrogen peroxide selects the helpers. In addition, it has been shown that there are toxic metabolites such as pyocyanin and rhamnolipids whose expression is QS-controlled and produced by the same cooperators which selectively affect the mutants; (García-Contreras et al. 2015, 2020); and a similar role has been attributed to hydrogen cyanide (HCN), a strong respiratory inhibitor produced maximally at low oxygen levels and whose production is upregulated by RhlR (Wang et al. 2015a, Yan et al. 2019). Nevertheless, this notion had been challenged by a study that found that in aerobic cultures used to study exoprotease cheating, HCN was not detectable and that defined *lasR* or *lasR rhlR* mutants are both equally affected as wild-type strains by HCN addition (Smith et al. 2019)**;** hence is not clear that HCN can act as a cheater policing metabolite. In addition to toxic metabolites, QS-deficient mutants are also more susceptible to the attack of some bacteriophages and their presence influences the population balance; cooperators can adapt to the cheating mutants by producing lower levels of exoprotease (hence becoming less exploitable) and higher levels of pyocyanin (restricting cheaters´ growth) (Saucedo-Mora et al. 2017, Castañeda-Tamez et al. 2018, Lai et al. 2018, Kramer et al. 2020). Interestingly, another function of exoproteases like AprA and LasB is to help *P. aeruginosa* avoid host immunity by degrading monomeric flagellin which is a ligand of TLR5 (Toll-like receptor 5) and which induces the production of cytokines such as IL-8 and NF-Kb (Casilag et al. 2015). Therefore, in principle, non-exoprotease producers could enjoy the benefits of immune evasion without paying the price of producing AprA; however, this has not been experimentally addressed yet.

The production of siderophores by *P. aeruginosa*, in particular pyoverdine, together with exoprotease production, is the most studied virulence factor for which cheating has been studied, usually by *in vitro* experiments in which iron is limited mostly by adding apo transferrin that chelates iron from the medium (Harrison et al. 2017). For this virulence factor, it has been demonstrated that its production is costly (Sexton and Schuster 2017), that laboratory-made mutants that do not produce it cannot grow well in iron-limited medium in monocultures, but can grow and increase their proportion and hence their fitness in the presence of siderophore producers (cooperators), and that their increase in fitness is inversely proportional to their initial frequency (Diggle et al. 2007, Kümmerli et al. 2015, Stilwell et al. 2018). In addition, mutants that do not produce pyoverdine but are able to assimilate it are often isolated from chronic pulmonary infections (De Vos et al. 2001, Jiricny et al. 2014, Andersen et al. 2015). And it has been suggested that they are selected due to social cheating of the siderophores; however, a plausible alternative is that these mutants accumulate because iron acquisition from the host is through heme groups and hence siderophores are not so important in these environments (Nguyen et al. 2014). Moreover, a detailed study made by Harrison and coworkers in 2017 revealed several potential failures in many of the experiments, including the use as a social cheater of a pyoverdine-deficient mutant with several additional mutations in several loci, including in genes of nitrate reductase, assimilation of nitrogen, QS, and motility (Harrison et al. 2017).

Furthermore, in order to acquire some insight into the possibility that pyoverdine cheating could potentially happen *in vivo*

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during infections, the authors performed experiments in growth media designed to mimic the conditions found in tissue and fluids of infected lungs of cystic fibrosis patients and in infections of non-healing wounds. They found that a pyoverdine mutant was less fit than the wild-type in artificial cystic fibrosis sputum, and found cheating pyoverdine production of the wild-type. In contrast, no effect of losing pyoverdine and hence no cheating was found in other media including *ex vivo* pig lung medium and two kinds of wound medium, one mimicking an acute and another a chronic wound infection (Harrison et al. 2017). Recently, we demonstrated that limiting the iron in the culture medium with apotransferrin not only imposes conditions in which pyoverdine is necessary for iron acquisition and growth but also promotes the loss and cheating of exoprotease production. This apparently unacknowledged property can be explained by the fact that since iron is bound to transferrin, its acquisition by *P. aeruginosa* is facilitated by the cleavage of transferrin by the exoproteases, making exoprotease synthesis susceptible to cheating. Hence, in order to avoid it, iron limitation should be implemented using non-proteinic chelating agents (Tostado-Islas et al. 2021). Nevertheless, it is likely that *in vivo* iron acquisition by *P. aeruginosa* is mediated by exoproteases and pyoverdine and hence, medium with iron restricted by transferrin may mimic the conditions more closely than medium in which iron is chemically removed.

The wide diversity of environments and hosts of *P. aeruginosa* affects the range of siderophores it can express (Kümmerli 2022). Experimental studies, as well as the isolation of strains in different environments, show that besides pyoverdine, *P. aeruginosa* expresses two other types of siderophores, pyochelin and pseudopaline. While pyoverdine and pyochelin are in charge of the exogenous acquisition of Fe, pseudopaline can also import Zi, Ni, and Co (Visca et al. 2007, Cornelis 2010, Youard et al. 2011, Ghssein and Ezzeddine 2022). Likewise, it has been determined that the expression of pyoverdine or pyochelin depends on the availability of Fe in the environment. Pyoverdine is expressed when the Fe concentration is low or its availability is difficult, for example, due to pH or temperature, whereas pyochelin is expressed when Fe limitation is not as strong (Dumas et al. 2013, Ross-Gillespie et al. 2015). The isolation of different strains under natural conditions, the use of nosocomial strains and experimental laboratory studies, have shown that mutants whose cellular and molecular structures can benefit from siderophores and do not synthesize and secrete them act as social cheaters. Although many aspects of the pyoverdine cheating phenomenon are known, the information regarding pyochelin cheaters has not been equivalent, despite the fact that it has been found that its presence is not redundant with pyoverdine and that the presence of one or the other siderophore is adaptive depending on the environmental context determined by Fe levels (Dumas et al. 2013). To the best of our knowledge, the study conducted by Ross-Gillespie et al. (2015) is the only one where the emergence of cheaters for pyochelin has been experimentally evaluated. To do this, the authors started from a strain (PAO1 *pvdD pchEF*) that was incapable of producing both pyoverdine and pyochelin. They found, as predicted, that in an environment with moderate Fe availability, pyochelin producers could be exploited by cheaters (Ross-Gillespie et al. 2015). These results are important because, in addition to corroborating the appearance of cheaters, both for pyoverdine and pyochelin, it was shown that different environments, with different populations of producers, were susceptible to being invaded by cheaters. To date, there are no studies regarding cheating of pseudopaline.

Besides the utilization of siderophores, pathogenic bacteria possess other strategies for capturing iron from their host; among them is the utilization of hemophores, which are proteins, secreted and used for heme scavenging, removing this iron-containing group from the host´s hemoproteins. Like siderophores, hemophores have extracellular receptors that bind them and allow heme internalization; once inside of the bacteria, the heme group is degraded and the iron released and assimilated, although there are some processes such as heme binding to the hemophore that do not consume energy, their production and export are metabolically costly (Cescau et al. 2007). Hence, hemophores seem to be public goods susceptible to cheating by bacteria that express the receptors; furthermore, It has been shown that heme uptake pathways in *P. aeruginosa* are upregulated by QS, particularly the expression of key proteins such as the hemophore receptor PhuR and the hemophore HasAp; in agreement, QS-deficient mutants do not grow well in media containing hemoglobin as sole iron source and the addition of QS signals restores this growth (Arevalo-Ferro et al. 2003).

Hence it seems plausible that naturally occurring QS-deficient *lasR* mutants have lower levels of hemophores and therefore behave as social cheaters of QS proficient/hemophore-producing individuals; nevertheless, no experimental studies exploring the susceptibility of heme acquisition to cheating are available yet.

## Biosurfactants

Swarming is a collective form of migration allowing bacteria to expand over soft surfaces and providing a group benefit. The secreted surfactants are potentially public goods susceptible to exploitation by surfactant-defectors which benefit from the surfactants secreted by others without producing surfactants themselves (Xavier et al. 2011). In *P. aeruginosa*, the main biosurfactants are rhamnolipids, amphipathic molecules that consist of a hydrophobic lipid and a hydrophilic sugar moiety. Rhamnolipids are tensioactives able to reduce surface tension, form emulsions, and promoting pseudosolubilization of insoluble substrates (Caiazza et al. 2005). In *P. aeruginosa*, rhamnolipids production occurs at the stationary growth phase and is transcriptionally regulated by QS (Ochsner and Reiser 1995) and post-transcriptionally modulated by the RsmA/RsmZ system (Heurlier et al. 2004). The biosynthesis of rhamnolipids in *P. aeruginosa* requires ketoacyl reduction by an Nicotinamide adenine dinucleotide phosphate (NADPH)-dependent B-ketoacyl reductase (RhlG) and obtains fatty acid from the general bacterial pool (Campos-García et al. 1998). The gene *rhlA* encodes the enzyme RlhA which produces 3-(3-hydroxyalkanoyloxy) alkanoic acids (HAAs), the lipid precursors of rhamnolipids. Downstream of *rhlA*, the genes *rlhB* and *rlhC* encode two enzymes, RhlB and RlhC; each one adds a rhamnose producing mono and di-rhamnolipids, respectively (Zhu and Rock 2008). The secreted surfactants are in principle public goods whose production can be exploited by surfactant-deficient defectors (Xavier et al. 2011). Rhamnolipids and rhamnolipid precursors produced by *P. aeruginosa* have many activities besides facilitating swarming motility, since they mediate the assimilation of hydrocarbons as nutrients (Beal and Betts 2000), change the biofilm architecture (Davey et al. 2003), have antibacterial properties (Haba et al. 2003) and disrupt host immunity during infections (Read et al. 1992). It is known that QS, rhamnolipids and bacterial appendages such as flagella and type IV contribute to swarming (Déziel et al. 2003). QS-mediated control of swarming is through RhlR, which activates the expression of the *rhlAB* genes (Ochsner et al. 1994). During swarming, *P. aeruginosa* secretes rhamnolipids, decreasing surface tension and allowing bacteria to move via flagellum-based propulsion (Kohler et al. 2000) by

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adopting a tendril shape. It is thought that rhamnolipids modulate swarming by allowing tendrils from different swarms to sense and respond to each other, and also by controlling the radial array tendrils in a single swarm (Caiazza et al. 2005).

Since rhamnolipids that are secreted by one bacterium can be also used by others, their production is an exploitable trait; but *P. aeruginosa* counteracts this by prudent regulation of rhamnolipids biosynthesis genes. Xavier et al. (2011) showed that *P. aeruginosa* regulates *rhlAB* expression to ensure that high secretions of rhamnolipids happen only when this will not severely impact bacterial growth. The regulation of biosurfactant synthesis in *P. aeruginosa* in the presence of excess carbon is closely tied to the growth rate and not just to cell density (as any regular QS-controlled trait). Hence, *P. aeruginosa* does not secrete biosurfactants and does swarm unless both QS and nutrient conditions are suitable.

On the other hand, recently it was demonstrated that rhamnolipids production selectively affects the growth of *lasR* mutants and can restrict social cheating, contributing to the maintenance of cooperation in *P. aeruginosa* populations (García-Contreras et al. 2020).

## Biofilms

Bacterial biofilms are three-dimensional structures composed of single or multiple bacterial species held together by extracellular matrix molecules that encapsulate bacteria allowing them to aggregate. Many bacteria that cause chronic infections rely on their ability to form biofilms for growth and proliferation. The extracellular polysaccharides (EPS) that are secreted by the bacteria are used as adhesins, able to attach cells to surfaces and contribute to the maintenance of biofilm structures; they also protect bacteria against several stresses like dehydration, antibiotics, and predators (Chiang et al. 2013, Thi et al. 2020). This extracellular matrix is composed of various elements including exopolysaccharides, proteins, and extracellular DNA (eDNA) that provide a protective element for the components of the biofilm. Because the biofilm provides so many benefits to the bacteria within it, the elements forming a biofilm are often costly for the members to produce and maintain.

## EPS and eDNA

Most of the matrix is composed of exopolysaccharides. These exopolysaccharides are matrix components that form the viscous, slime-like layer characteristic of a biofilm. There are three main exopolysaccharides that contribute to the biofilm: Psl, Pel, and alginate. These substances provide the bacteria mainly with a protective layer against antibiotics and other potential threats to the community. Alginate is the main contributor to biofilms in pathogenic strains of *P. aeruginosa*. Alginate is composed of $\alpha$-D-mannuronic acid and gluronic acid. The production of this copolymer provides the colonies with a mucoid phenotype (Ghafoor et al. 2011). The overproduction of this exopolysaccharide often contributes to the pathogenicity of *P. aeruginosa* in Cystic Fibrosis patients and provides protection against antibiotics. Another important component of the matrix is eDNA, since it provides the biofilm with structural strength and nutrition during times of starvation, and also enables horizontal gene transfer (Ibáñez de Aldecoa et al. 2017). When eDNA is combined with Psl, thick rope-like structures surround cell aggregates. This interaction between eDNA and Psl assists the biofilm with adhesion to surfaces, as well as offering structural support and antibiotic resistance (Wang et al. 2015b).

There are different mechanisms enabling DNA to enter the extracellular matrix. While some cells actively secrete DNA, others undergo autolysis when the population reaches a certain density. Because these Psl-DNA fibers have been found around dead cells, it is thought that a lot of eDNA is derived from lysed cells within the biofilm. This mechanism is generally a result of QS signals. When cell populations reach a certain density, cooperative behaviors such as eDNA production begin to be regulated by QS (Ibáñez de Aldecoa et al. 2017). *P. aeruginosa* will mediate cell lysis and/or active secretion of DNA from some cells when it benefits the community. It will mediate this process through the Pseudomonas quinolone signal (PQS) QS system (Ibáñez de Aldecoa et al. 2017).

## Social cheating of EPS and eDNA in biofilms

Biofilms are an important aspect of bacterial growth since bacteria use these extracellular matrices to attach to surfaces, communicate with each other and share "public goods." These collective actions benefit the bacterial community. However, this provides the perfect opportunity for cheaters to take advantage of members producing these structural components while not contributing to the biofilm. In this case, cheaters will enjoy the benefits of the biofilm without having to contribute to its formation and maintenance, weaken the biofilm overall (Popat et al. 2012). The biofilm and its components are costly for cooperative bacteria to produce; however, the benefits are crucial to the proliferation of bacterial communities. Cheaters enjoy the many benefits of these components but decrease the strength, antibiotic resistance, and sharing of public goods among cooperative members. Hence is interesting to determine how the production of these diffusible public goods of the biofilm can remain stable against cheaters. It is now understood that cheaters only reap the benefits of these goods within a close spatial range. The spatial range around producers is quantifiable and limited to areas surrounding cooperators. These spatial ranges are dependent on rates of diffusion of the public goods (Tai et al. 2022).

The stable production of EPS is a potential evolutionary problem because it seems to be a cooperative behavior providing a benefit to all bacteria in the population and not just to those cooperators that produce it. Hence, it is interesting to understand what prevents the invasion of biofilms by cheaters maintaining the EPS secretion functional not only in *P. aeruginosa* but in related species such as *P. fluorescens* (Rainey and Rainey 2003). Such social cheaters would have a fitness advantage, exploiting the benefits of the EPS produced by the cooperators without paying the price of its production. The biofilm matrix in *P. aeruginosa* is composed of at least three different EPS molecules, alginate, Psl and Pel, and eDNA (Wei and Ma 2013). It has been shown that although the production of Psl in its biofilms is a social trait that provides benefits at the individual and group level, it cannot be successfully cheated on by defectors (Irie et al. 2017). This is so even though the Psl non-producer strain can benefit from the presence of the Psl-producing cooperators in biofilms. This occurs since the benefits of producing Psl favor mainly the producing bacteria or other Psl-producing bacteria. This is different in regard to other social traits in *P. aeruginosa*, such as the production of siderophores and exoproteases which are readily exploited by cheaters *in vitro* and *in vivo* biofilms (Griffin et al. 2004, Kümmerli et al. 2015).

Likely this is because siderophores and most QS-dependent public goods are freely diffusible. In contrast, the primary function of EPS such as Psl is to assist bacteria with adherence to surfaces and to adhere to other bacteria; Hence, Psl has a very limited diffusion within biofilms. Consistent with this, Psl localizes prefer-

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entially in the periphery of biofilm microcolonies, encapsulating the cells (Borlee et al. 2010). In addition, some Psl is closely associated with the bacterial surface (Ma et al. 2006), although a fraction of Psl is more diffusible and serves as an intercellular signaling molecule (Irie et al. 2017).

The signaling properties of Psl coordinate changes in gene expression at the community level, increasing its own production (hence being auto-inducible) as well as the production of other biofilm-associated factors (Irie et al. 2017). To date, no studies addressing the exploitability of the other two important biofilm EPS, Pel and alginate, by defectors are available.

Other components of biofilms such as diffusible biofilm matrix proteins are susceptible to cheating and the stability of their production depends on biofilm spatial structure, the sharing mechanisms of these biofilm matrix components, and in the flow conditions (Tai et al. 2022). Hence, exploitation of diffusible adhesion proteins is possible only at a short spatial range around cooperators, allowing that the production of diffusible biofilm matrix proteins remains stable in natural biofilm habitats (Tai et al. 2022). The size of the spatial range that allows the exploitation of diffusible matrix components depends on the rates of diffusion and advection within the biofilms. Hence, a sparse distribution of bacterial clusters and the environmental flow rate lower the exploitation of these components (Tai et al. 2022). This property allows the stability of *P. aeruginosa* and many other bacterial biofilms in natural habitats and is supported by social evolution theory, according to Hamilton´s rule (Tai et al. 2022).

The eDNA secreted during bacterial lysis contributes to biofilm growth (Bayles 2007). Other than benefitting the long-term success of the bacterial population, bacterial lysis does not provide a clear advantage. Hence, it is important to understand whether a selective advantage for *P. aeruginosa* to induce autolysis exists. One possibility is that it may promote biofilm formation and antimicrobial tolerance, and another is that it may facilitate the release of nutrients that can be used for the survival of the remaining population.

In *P. aeruginosa*, autolysis is regulated by QS; for example, it is well established that *lasR* mutants avoid lysis in planktonic cultures (Heurlier et al. 2005) and that quinolone-mediated QS promotes a kind of programmed cell death by poisoning the respiratory chain via the respiratory inhibitor 2-heptyl-4-hydroxyquinoline-N-oxide (HQNO) which induces the production of reactive oxygen species (ROS). This allows the release of eDNA, increasing biofilm formation and antibiotic tolerance (Hazan et al. 2016). Moreover, QS-controlled toxic compounds such as pyocyanin also increase oxidative stress, facilitating eDNA release and favoring biofilm formation (Meirelles and Newman 2018). Although the effects of bacterial lysis and eDNA release seem to benefit the population, it is not clear to what extent social cheating may counteract those benefits, since it seems straightforward that if a fraction of the population is less prone to lysis (for example, *lasR* mutants) they will cheat the cooperators increasing their fitness. On the other hand, there may be some mechanisms that restrict the possible harm of cheating, for example, a spatial structure clustering the cooperators so the benefit of cell lysis is provided locally (Kümmerli et al. 2009), or policing mechanisms such as the effect of compounds like pyocyanin (Castañeda-Tamez et al. 2018), rhamnolipids (García-Contreras et al. 2020), etc., which may increase the frequency of lysis in potential cheaters such as QS deficient strains.

Another factor that influences biofilm formation and its architecture is bacterial motility; for example, in *P. aeruginosa* swarming determines biofilm architecture. Also, available carbon sources exerts control over the surface motility and QS influencing the initial coverage of the substratum by founder biofilm bacteria (Shrout et al. 2006); hyper-swarming motility produces flat and uniform biofilms, whereas lower swarming leads to bacterial aggregates and the formation of microcolonies, hence it may be possible that hyper swarming increases the probability of social cheating by homogenizing the environment, whereas low-swarming motility counteracts it by promoting spatial organization, increasing the mean distance between cooperators and potential cheaters.

## Secretion systems

Bacteria employ sophisticated protein secretion mechanisms to colonize their niches and compete for resources. In Gram-negative bacteria, eight protein secretion systems have been described, referred to as “Type "X" secretion system (TXSS)” for Type “X” secretion system, where “X” is the number corresponding to each system (1–6, 9, and 10) (Denise et al. 2020). The secretion of substrates through these molecular machineries can be classified into two different categories: one-step secretion when proteins are transported directly from the bacterial cytosol to the extracellular space without periplasmic intermediates as in the T1SS; moreover, in the T3SS, T4SS, and T6SS, substrates reach the cytosol of other target cells, Bacteria or Eukarya. The substrates in two-step secretion are first exported to the periplasm through the Sec translocase or Twin-arginine translocation pathway, then the proteins are secreted to the extracellular space as in the T2SS, T9SS, and T10SS, or inserted into the outer membrane, as is the case for T5SS (Grossman et al. 2021). In this section, we review the available information about cheating on different protein secretion systems and their implications for the bacterial community.

Although secretion systems are multi-protein complexes attached to the bacterial cells that produce them and are therefore private goods, the benefits exerted by their effectors can in principle be enjoyed by both individuals that produce the systems and secrete the effectors, and by those that do not; hence, exploitation of these systems could be possible. Another condition needed for a factor to be susceptible to cheating is that its production should be metabolically costly, and for secretion systems, this is the case since making the proteins requires high levels of Adenosine triphosphate (ATP), as well as the energy for protein translocation across T3SS, T4SS, and T6SS, which is provided in the first instance by ATP hydrolysis from ATPases dedicated to each system (Díaz-Guerrero et al. 2018). This suggests that the assembly and function of these molecular machines would have a metabolic cost that could compromise the ATP reserves used by bacteria for biomass production and cellular maintenance. In this regard, it was estimated that *Vibrio fischeri* spend between 3.1% and 13.6% of their free ATP to assemble and secrete proteins through their T6SS (Septer et al. 2023). The T6SS is not exclusive to *V. fischeri,* but is present in nearly 25% of Gram-negative pathogens, including *P. aeruginosa*, *Burkholderia mallei*, and *Campylobacter jejuni,* etc. This is a versatile secretion machinery that, in addition to carrying out the elimination of competitors in the environment, is also involved in target cell adhesion and invasion. In a co-culture competition assay it was observed that a *C. jejuni* wild-type strain has a functional T6SS (producer) able to eliminate *Escherichia coli* DH5a, while a T6SS negative mutant (cheater strain) was not able to do this. However, the *C. jejuni* population that maintained an active T6SS became extinct faster than *E. coli* and T6SS null mutant, under conditions of environmental stress. While the T6SS may gain an ecological advantage by

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eliminating competitors, this clearly carries a metabolic cost. Maintaining environmental stress favors the probability of survival of cheater bacteria lacking T6SS, which would allow them to dominate the ecological niche once bacteria with active SST6 eliminates competitors before population collapse (Gupta et al. 2021).

Something similar was observed in mouse co-infection assays with *P. aeruginosa* strains expressing (producer) or not T3SS (cheater). *Pseudomonas aeruginosa* utilizes ExoU, a T3SS effector, to inhibit the neutrophil-mediated host immune response. In these conditions, *P. aeruginosa* T3SS cheaters outcompete the wild-type strain; this confers competitive advantage over producers since ExoU is a public good and hence, avoiding its production provides the non-producers a fitness advantage by not incurring the energy waste needed to assemble and secrete proteins through the T3SS; thus, although T3SS mutants do not secrete ExoU, they benefit from its secretion by the wild-type bacteria. At the earlier stage of bacterial infection, *P. aeruginosa* with active T3SS outnumber neutrophils recruited to the infection site and T3SS mutants. The ExoU-mediated immunosuppression by the wild-type strain would facilitate higher cheater replication, allowing the latter to establish a persistent infection and facilitating their enrichment over wild-type individuals in acutely infected patients (Czechowska et al. 2014).

Given this scenario, one question arises: If cheaters have a competitive advantage over producers, why do cheaters not completely eliminate producers? The obvious answer would be that the cheaters' survival depends on the public good produced by cooperative bacteria. In this regard, several hypotheses have arisen. Here we will discuss two of the most interesting. The first proposes that cheaters can establish a mutualistic relationship with producer bacteria under certain ecological conditions (García-Contreras and Loarca 2021). For example, a two-component system GacA/GacS mutant of *P. chlororaphis* is unable to produce biofilm, but in mixed liquid cultures overcomes the wild-type strain. Interestingly, when both strains were co-cultured in conditions of biofilm induction, the mixed cultures were more efficient than the wild-type monoculture, indicating that the presence of cheaters is somehow beneficial to the wild-type strain, highlighting the importance of environmental conditions of the bacterial community (Driscoll et al. 2011). The second hypothesis also highlights the importance of the environmental context and was found in mixed cultures of *Burkhorderia thailandensis*. When the wild-type strain and a QS mutant are growing in liquid coculture, the QS mutant outcompetes the wild-type strain, a typical behavior for cheaters. However, when both strains were switched to agar plates, the cheater advantage disappeared, indicating that the wild-type strain can inhibit cheater growth. Transcriptomics data in *B. thailandensis* revealed that T6SS encoding genes are regulated by QS and a competition assay between a T6SS mutant and a QS mutant showed that QS cheaters regained the growth advantage on solid medium over the T6SS mutant. These results indicate that the T6SS of *B. thailandensis* functions as a bacterial policing mechanism that could inhibit the proliferation of QS cheaters, providing producers with a kind of immunity against cheaters (Majerczyk et al. 2016).

## Pyocyanin

Pyocyanin is one of the most important virulence factors produced by *P. aeruginosa*. This is an active redox phenazine that can reduce $O_2$-producing ROS such as hydrogen peroxide, superoxide, and hydroxyl radical. These ROS increase the oxidative stress in both prokaryotic and eukaryotic cells, leading to their detriment and finally to their death (Hassett et al. 1991, Hall et al. 2016). Thus, this metabolite provides an advantage against other microorganisms inhibiting their growth. Moreover, mutant strains unable to produce pyocyanin also are attenuated in their virulence (Lau et al. 2004). Since pyocyanin is a toxic compound, *P. aeruginosa* orchestrates a protective response to avoid its damage; this response includes a marked stimulation in catalase production, alkyl hydroperoxide reductases, efflux pumps, and SOS response, among others (Hassett et al. 1991, Dietrich et al. 2006, Meirelles and Newman 2018).

Pyocyanin production is tightly regulated at the transcriptional level by the QS cascade that involves the Las, Rhl, and PQS systems. These three QS systems control the expression of the two redundant operons, *phzA1-G1* and *phzA2-G2*, involved in pyocyanin synthesis. Previously we have shown that pyocyanin is able to restrict cheating when grown in the presence of *P. aeruginosa* PA14 mixed with a *lasR rhlR* double mutant strain in M9 caseinate medium, using proteases production as an indicator of cheating. Results showed that the percentage of the double mutant strain increased until 10 h and then it remained stable, indicating that the double mutant can grow initially by using the proteases produced by the wild-type strain, acting as cheaters. However, the production of a toxic metabolite such as pyocyanin, produced by the wild-type strain after 10 h, avoids increasing cheaters. This effect is not observed in co-cultures using a *phzM* mutant strain, unable to produce pyocyanin, and the *lasR rhlR* double mutant strain, but it is restored when pyocyanin is added to the medium. These results indicate that this virulence factor is able to restrict cheating in these conditions. However, it cannot be ruled out that in other conditions pyocyanin can be used as a public good. For example, it has been demonstrated that the protective response to avoid self-poisoning by pyocyanin is mediated by the transcriptional factor SoxR (Dietrich et al. 2006). Thus, it is possible that social cheaters can use pyocyanin to activate SoxR and therefore, the protective response. Also, this response in social cheaters can allow them to grow and to compete by niche since pyocyanin is detrimental to other bacteria (Hassan and Fridovich 1980). Besides the toxic effects of pyocyanin, in *P. aeruginosa* this virulence factor is involved in maintaining the balance between Nicotinamide adenine dinucleotide hydrogen (NADH) and Nicotinamide adenine dinucleotide (NAD)+ in anoxygenic conditions, acting as an electron acceptor (Price-Whelan et al. 2007). Thus, social cheaters can also use pyocyanin to maintain the redox balance, allowing them to grow when oxygen is consumed.

Moreover, pyocyanin also aids *P. aeruginosa* in iron (Cox 1986) and phosphorus (Mcrose and Newman 2021) acquisition, and counteracts the toxic effect of metals such as silver (Muller and Merrett 2014) and gallium (García-Contreras et al. 2013); thus, in principle, non-pyocyanin producers may act as social cheaters exploiting cooperators in conditions of low iron or phosphorus availability and in the presence of gallium or silver. Finally, pyocyanin can enhance biofilm formation by a mechanism not totally understood, but it has been reported that the auto-poisoning caused by pyocyanin allows eDNA release which is important for biofilm formation (Das and Manefield 2012). Thus, social cheaters can use pyocyanin to promote their survival when biofilm is formed.

## Conclusion

*Pseudomonas aeruginosa* presents a plethora of virulence factors that allow successful colonization of their host and the establishment of acute and chronic infections; nevertheless, many of

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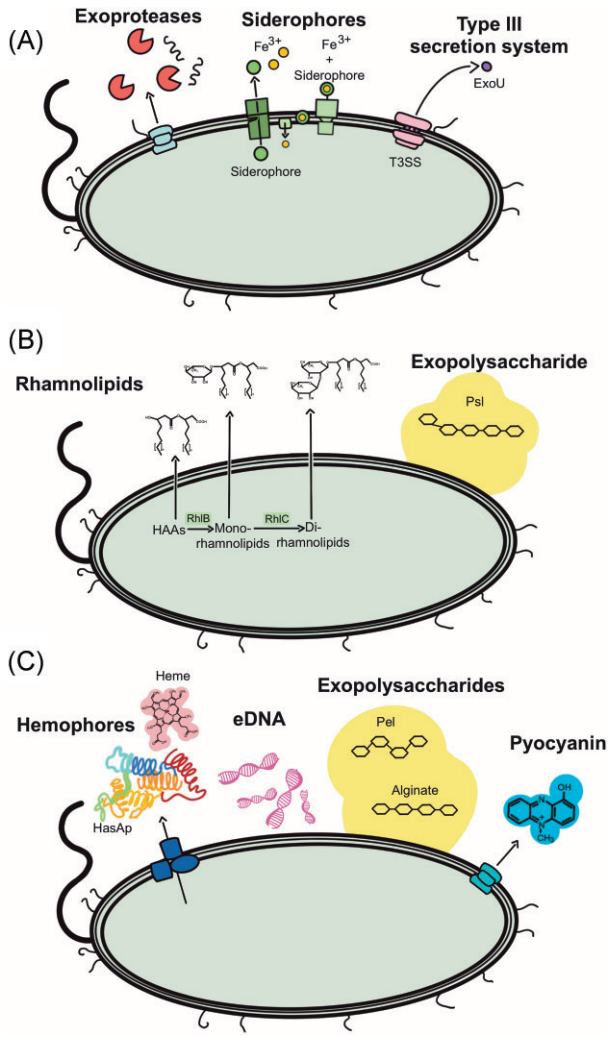


**Figure 1.** (A) Virulence factors strongly susceptible to social cheating, (B) Virulence factors weakly susceptible to social cheating, and (C) Virulence factors for which is unknown if they are susceptible to exploitation by defectors.

the virulence factors produced by this opportunistic pathogen are public goods, and hence in principle susceptible to social cheating to some degree. Current evidence demonstrate that some factors such as exoproteases, siderophores and the T3SS effector ExoU are cheatable (Fig. 1A), while others such as rhamnolipids and Psl EPS are not so susceptible to cheating (Fig. 1B) due metabolic prudence in the case of the first and low diffusion in the case of the second; finally, information about cheating susceptibility for other virulence factors such as hemophores, pyocyanin, Pel, alginate, and eDNA is scarce (Fig. 1C) and hence no conclusions can be drawn yet, representing a possible research area for future studies. We also encourage the study of the consequences of social cheating during *P. aeruginosa* infection, as well as the design and implementation of therapeutic alternatives based on the modulation of this social behavior.

## Author contributions

Katya Dafne Guadarrama-Orozco (Conceptualization, Writing – original draft), Caleb Perez-Gonzalez (Conceptualization, Writing – original draft), Kokila Kota (Conceptualization, Writing – original draft), Miguel Cocotl-Yañez (Conceptualization, Writing – original draft), Jesús Guillermo Jiménez-Cortés (Conceptualization, Writing – original draft), Miguel Díaz-Guerrero (Conceptualization, Writing – original draft), Mariel Hernández-Garnica (Conceptualization, Writing – original draft), Julia Munson (Conceptualization, Writing – original draft), Frederic Cadet (Conceptualization, Writing – original draft, Writing – review & editing), Luis Esaú López-Jácome (Conceptualization, Writing – original draft), Ángel Yahir Estrada-Velasco (Conceptualization, Writing – original draft), Ana María Fernández-Presas (Conceptualization, Writing – original draft), and Rodolfo García-Contreras (Conceptualization, Supervision, Validation, Writing – original draft, Writing – review & editing)

*Conflict of interest*: We have no conflict of interest.

## Funding

The work of R.G.-C. was supported by DGAPA, PAPIIT-UNAM under grant number [IN200121] and M.D.-G. was supported by a postdoctoral DGAPA UNAM grant.

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**Received** 23 July 2023; **revised** 30 August 2023; **accepted** 11 October 2023
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