

Exoprotease exploitation and social cheating in a *Pseudomonas aeruginosa* environmental lysogenic strain with a non-canonical quorum sensing system

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One sentence summary: The *lasR*-deficient ID4365 *P. aeruginosa* strain is subject to exoprotease exploitation, *rhlR* mutants behave as social cheaters, and the production of temperate phages may accelerate population collapses.

Abstract

Social cheating is the exploitation of public goods that are costly metabolites, like exoproteases. Exoprotease exploitation in *Pseudomonas aeruginosa* has been studied in reference strains. Experimental evolution with reference strains during continuous growth in casein has demonstrated that non-exoprotease producers that are *lasR* mutants are selected while they behave as social cheaters. However, non-canonical quorum sensing systems exist in *P. aeruginosa* strains, which are diverse. In this work, the exploitation of exoproteases in the environmental strain ID4365 was evaluated; ID4365 has a nonsense mutation that precludes expression of LasR. ID4365 produces exoproteases under the control of RhlR, and harbors an inducible prophage. As expected, *rhlR* mutants of ID4365 behave as social cheaters, and exoprotease-deficient individuals accumulate upon continuous growth in casein. Moreover, in all continuous cultures, population collapses occur. However, this also sometimes happens before cheaters dominate. Interestingly, during growth in casein, ID4565's native prophage is induced, suggesting that the metabolic costs imposed by social cheating may increase its induction, promoting population collapses. Accordingly, lysogenization of the PAO1 *lasR* mutant with this prophage accelerated its collapse. These findings highlight the influence of temperate phages in social cheating.

Keywords: Quorum sensing, social cheaters, RhlR, exoprotease, temperate phages, population collapses

Introduction

Production of public goods such as siderophores and exoenzymes by cooperating bacteria is susceptible to exploitation by non-producers that enjoy their benefits without investing in their synthesis. Such individuals are considered social cheaters, and if they accumulate to high levels, the growth of the population can collapse due to overexploitation in a tragedy of the commons (Smith and Schuster 2019).

Studies on social cheating of exoprotease production in bacteria have mainly been conducted in *P. aeruginosa* reference strains PAO1 and PA14, with only few reports using clinical strains (Loarca *et al.* 2019), and no studies have been done using environmental strains. Therefore, the study of social cheating from a greater diversity of *P. aeruginosa* strains could help to better understand the dynamics of this behavior, the molecular genetic bases, and the potential mechanisms that bacteria use for its regulation. According to what is known about the PA14 and PAO1 strains, population collapse occurs when exoprotease non-producer cheaters accumulate to a high proportion of the population, since the remaining cooperators are unable to produce enough exoprotease to maintain their own growth due to overexploitation (Wilder, Diggle and Schuster 2011; Dandekar, Chugani and Greenberg 2012; Castañeda-Tamez *et al.* 2018; Loarca *et al.* 2019). Interestingly, factors such as a downregulation of the production of exoprotease by cooperators (Lai *et al.* 2018) and the quorum sensing (QS) controlled synthesis of toxic metabolites that preferentially inhibit the growth of the cheaters, such as pyocyanin and rhamnolipids (Castañeda-Tamez *et al.* 2018; García-Contreras *et al.* 2020), can stabilize the populations, leading to a long-

lasting equilibrium between cooperators and cheaters. The production of toxic metabolites like HCN has been also proposed as a mechanism to punish cheaters (Wang *et al.* 2015); however, its role remains controversial since it is not produced to high concentrations upon aerobic growth (a condition used for studying exoprotease exploitation) and evidence suggests that the molecular mechanisms related to HCN tolerance are not QS-controlled (Smith *et al.* 2019).

Since the expression of exoproteases and many virulence factors in *P. aeruginosa* reference strains are strongly dependent on the main QS receptor LasR, virtually all the exoprotease cheaters obtained in evolution experiments with continuous growth in media with protein as sole carbon or carbon/nitrogen source, are mutants that lack LasR activity (Sandoz, Mitzimberg and Schuster 2007; Dandekar, Chugani and Greenberg 2012; Loarca *et al.* 2019). However, at least for the PAO1 reference strain, LasR plays no significant role in QS in conditions such as growth in low phosphate, and it is replaced by the RhlR regulator (Soto-Aceves *et al.* 2020). Furthermore, it is known that some clinical (Cruz *et al.* 2020) and environmental (Martínez-Carranza *et al.* 2020) strains still produce QS-dependent virulence factors in the absence of a functional LasR. Among them is the strain ID4365 that has a nonsense mutation in *lasR* and is unable to produce a functional LasR regulator. In this strain, the production of QS traits such as pyocyanin and exoproteases is controlled by RhlR (Cocotl-Yañez *et al.* 2021). Hence, it is interesting to determine if mutants with an inactivated RhlR would behave as exoprotease cheaters that can be selected with continuous growth in casein as the sole carbon source.

Moreover, due to the ubiquity of bacteriophages and their intimate relationship with their host, it is likely that most of the bacterial strains in the environment carry active prophages (Srividhya *et al.* 2007; Johnson, Banerjee and Putonti 2022; Aledo *et al.* 2023) that enter the lytic phase either stochastically or when stressed. Also, the possible role of the prophages in exoprotease exploitation is not yet explored. For the strain ID4365, genomic analysis revealed the existence of one putative complete prophage of 58.3 kb, and we determined that indeed this strain produces phage particles upon its cultivation in caseinate media. Hence, in this work, we studied the growth dynamics, exoprotease production, and prophage induction in the strain ID4365 during continuous growth in caseinate as the sole carbon source.

Our findings demonstrate that in strains like ID4365, *rhlR* mutants behave as social cheaters and that exoprotease-deficient individuals can be selected upon continuous cultivation in casein as the sole carbon source. However, unlike reference strains, the accumulation of these low exoprotease producers is not the only factor contributing to population collapses, as they can be observed even when there is not a high proportion of exoprotease-deficient individuals. Since we found ID4365 harbors an inducible podovirus highly produced during casein cultivation, we investigated if its induction was linked to the observed collapses by transferring this temperate phage to a PAO1 *lasR* mutant background. In agreement with our hypothesis, the presence of this F116-Like temperate phage accelerated population collapses upon exoprotease exploitation in this reference strain. Hence, our study increases the understanding of factors that influence the outcomes of cheating as well as an understanding of alternative ways to produce exoprotease social cheaters.

Materials and methods

Strains and growth conditions

The wild-type strain used in this work is ID4365, an environmental strain isolated from the Indian Ocean (Manwar *et al.* 2004), with a nonsense mutation in *lasR* that changes the CAG codon at amino acid 24 to a TAG stop codon. Hence, this strain controls the expression of QS dependent virulence factors, such as pyocyanin and elastase, through RhlR (Cocotl-Yañez *et al.* 2021). In addition, we used the ID4365 *rhlR* isogenic mutant (Cocotl-Yañez *et al.* 2021), PAO1, PAO1 *lasR* (Soto-Aceves *et al.* 2020), the PAO1 *lasR* ΦIDHM phage lysogenic (this work), and PAΦ, an environmental strain isolated from a natural hot spring highly sensitive to phages. Protease-deficient clones derived from continuous growth in casein from ID4365, PAO1 *lasR*, and the PAO1 *lasR* ΦIDHM lysogen were electroporated with the plasmids pUCP20 or pGMYC (pUCP20-*rhlR*) (Medina *et al.* 2003), and the plasmids were selected with carbenicillin 200 µg/ml.

Strains were routinely cultured in LB or BHI media at 37°C. For the competition and evolution experiments, M9 minimal media with 0.25% caseinate as the sole carbon source was used.

Exoprotease and pyocyanin determination

Exoprotease (caseinolytic activity) was determined either qualitatively by growing colonies on plates with M9 media with casein and casamino acids as carbon sources and evaluating the presence of a clear halo produced due casein degradation or quantitatively by measuring the azocasein degradation in the supernatants of the bacterial cultures (Loarca *et al.* 2019) grown either in LB or BHI media. Pyocyanin from the supernatants was extracted with chloroform and extracted again with 0.2 N HCl, and its absorbance at 520 nm was determined for its quantification as previously described (Essar *et al.* 1990).

Bacterial competition experiments

Competition experiments were performed by inoculating cultures of M9 caseinate medium with the ID4365 wild-type strain (exoprotease producer) and its isogenic mutant ID4365 *rhlR* with initial proportions of approximately 95% and 5%, respectively. The proportions of each strain were calculated using the turbulence 600 nm values of overnight cultures, and experiments were performed in duplicate. To determine the strain proportions at the beginning of the experiment, an aliquot of the cultures was streaked on LB plates, incubated overnight, and single colonies were transferred to M9 casein/casamino acid plates, which were incubated overnight and assessed for the visualization of exoprotease production, to determine the initial proportions of the competitor strains. The mixed cultures were then incubated at 37°C with 200 rpm shaking, and aliquots were taken at 24 and 48 h and used for the determination of the strain proportions.

Evolution experiments

Single colonies of the different strains were used to inoculate overnight cultures made in BHI medium, that later were used to begin growth passages in M9 caseinate liquid medium. Each pass was initiated at a turbulence of 0.05 and cultured at 37 °C and 200 rpm shaking for 48 h. For the inoculation of a new growth passage at turbulence 0.05, the needed amount of cells were obtained, washed with sterile 0.9% NaCl solution and used to inoculate the culture for the next growth passage. After each growth passage, 10 colonies per culture

were isolated and cultured in LB to determine their exoprotease production. In addition, the supernatants were filtered and also used to determine and quantify bacteriophage production.

Genomic DNA extraction, PCR, and complementation

Genomic DNA of ID4365, PAO1 *lasR* and the PAO1 *lasR* Φ IDHM lysogen were extracted using the Quick-DNA™ miniprep plus kit, and PCR was used to amplify *csrA*, which is only present in the Φ IDHM phage genome. Oligonucleotides to amplify *csrA* were: forward, 5'-GTGACCGTCGTCGAGAACAGA-3'; reverse, 5'-GCCTGACCTTCTAGCCAGATC-3', and the conditions of PCR were 95 °C, 1 min then 29 cycles: 95 °C, 1 min; 60 °C 30 s; 72 °C, 30 s; final extension step, 72°C, 5 min.

Protease-deficient clones derived from the strains ID4365, PAO1 *lasR*, and the PAO1 *lasR* Φ IDHM lysogen were transformed with pGMYC (pUCP20-*rhlR*) or with the empty pUCP20 vector using electroporation and selected with LB carbenicillin 200 µg/ml plates. After selection, colonies were isolated and transferred to new LB carbenicillin plates, cultured in LB carbenicillin, and the supernatants were used for pyocyanin and caseinolytic activity.

Whole genome sequencing

The procedures for the whole genome sequencing of two non-exoprotease producers derived from ID4365 were those described in Tostado-Islas *et al.* 2021. Briefly, genomic DNA was extracted using the DNeasy UltraClean Microbial Kit (Qiagen, Hilden, Germany), the library was formed using the Nextera XT DNA Sample Prep Kit with TruSeq HT adapters (Illumina, San Diego CA, USA) for barcoding, and libraries were sequenced with an Illumina MiSeq instrument using Trimmomatic v.0.39 to remove sequencing adapters and bad-quality sequencing data (Bolger, Lohse and Usadel 2014).

Bioinformatic analysis

The genome of the ID4365 native phage was identified from the genome sequence of the ID4365 strain using PHASTER. The visualization and comparison of Φ IDHM's genome and its homologous was generated using the GView server (<https://server.gview.ca/>) (Petkau *et al.* 2010). We searched for single nucleotide polymorphisms (SNPs), insertions, and deletions in the evolved exoprotease deficient strains ID14 and ID16, comparing them to the parental ID4365 genome (Grosso-Becerra *et al.* 2014). From the ID4365 genome, we extracted the *rhl* operon (*rhlA-rarD*) and utilized it to identify SNPs in the genomic assemblies of both ID14 and ID16. This analysis was performed using nucmer (Marçais *et al.* 2018).

To ensure the conservation of mutations across pseudomonads, we downloaded the representative PAO1 genome and the following genomes from GenBank: GCA_001457615.1_NCTC10332, GCA_900095805.1_PA14Or, GCA_900149285.1_PcyII-10, GCA_900185255.1_NN2, GCA_900243355.1_RW109, GCA_900618245.1_paerg000, GCA_900618255.1_paerg002, GCA_900618265.1_paerg004, GCA_900618275.1_paerg003, GCA_900618285.1_paerg005, GCA_900618305.1_paerg009, GCA_900618315.1_paerg010, GCA_900618325.1_paerg011, GCA_900618335.1_paerg012, GCA_900636735.1_43941_C01, GCA_900636755.1_43941_E01, GCA_900636975.1_45813_F01, GCA_900637045.1_46837_C02, GCA_902172305.2_*Pseudomonas aeruginosa* PAKAF, GCA_902703185.1_PcyII-29, GCA_902703195.1_PcyII-40, GCA_902703215.1_C7-25, GCA_904866275.1_MINF_7A, and GCA_905071885.1_MINF_3A. These genomes were analyzed by parsnp, part of the Harvest suite (Treangen *et al.* 2014) to calculate a core genome tree and both *rhl* mutations and whole-genome level substitutions were examined.

Phage titer determination

Phage titers were determined by centrifuging 1 mL aliquots at 13,000 rpm for 5 min, filtering the supernatants (MF-Millipore membrane filter, 0.22 µm pore size) and dropping 5 µL of serial dilutions on PAΦ lawns in a double overlay plaque assay. TΦ media was used for the bottom agar (1% peptone, 0.5% NaCl, 1.1% Bacto agar) and the same media with 0.7% of Bacto agar was used for the top agar (Kropinski *et al.* 2009). Three to 30 plaques were considered as countable.

Electron microscopy

Ten microliters from a phage suspension of $\sim 10^{10}$ PFU/mL were deposited on a collodion-coated copper grid and incubated for 5 min at room temperature. The excess solution was adsorbed with filter paper and the grid was stained once with uranyl acetate (2%, pH 7) for 1 min. Grids were examined under a Jeol JEM 1010 transmission electron microscope at 80 Kv. Dimensions of the virions were calculated from the average of 10 viral particles.

Lysogenization of PAO1 *lasR* with the ΦIDHM phage

The turbid zone from a well-separated plaque on a bacterial lawn of PAO1 *lasR* was picked with a sterile toothpick and streaked on LB agar. Twenty-three colonies were selected and purified by streaking each of them three times. Single colonies of each candidate were grown overnight and diluted to a turbulence of 0.05, then grown to a turbulence of ~ 0.5 , then, mitomycin C was added at a final concentration of 3.0 µg/mL. After 6 hours of incubation, 1 mL of the culture was centrifuged at 13,000 rpm for 5 min, and 10% of chloroform was added to the supernatants. Serial dilutions at 1:100 were performed and

dropped on a lawn of the parental strain of PAO1 *lasR*. The presence of lytic plaques indicated that candidate colonies may be lysogens. To confirm this, an assay of hom immunity was performed: lawns of each possible lysogen were assayed, and dilutions of Φ IDHM phage were dropped. After incubation at 37°C, overnight, if there were no lytic plaques, the assay was positive. To confirm lysogens, a PCR for the *csrA* gene, present only in the genome of Φ IDHM phage, was performed.

Results

Natural isolate ID4365 (*LasR* inactive) can grow using caseinate as the only carbon source

Previously it was reported that ID4365 harbouring a nonsense mutation that inactivates *lasR* had a significant but low elastolytic activity controlled by RhlR (Cocotl-Yañez *et al.* 2021). Consistent with this, we found that the caseinolytic activity of ID4365 was considerably lower than the activity observed in PAO1. Nevertheless, the ID4365 grew with caseinate as the sole carbon source, although slower than PAO1 (Figure S1A).

ID4365's *rhlR* mutants behave as social cheaters

To investigate whether a *rhlR* mutant of ID4365 behaves like a social cheater, its growth in M9 medium with caseinate as the sole carbon source was compared with the growth of the ID4365 wild-type strain, and, as expected, its growth was severely impaired. In contrast, the growth of both strains in the same medium but replacing the caseinate with casamino acids, was very similar (Figure S1B). Hence, we hypothesized that in mixed cultures in casein as the sole carbon source, the ID4365 *rhlR* strain would grow at the expense of the protease produced by the wild-type. Therefore, to evaluate this, competition assays were conducted between this mutant and its parental strain in casein media. The initial proportion of the mutant population increased from 6 ± 3 % to 30 ± 3 % and 36 ± 6 % after 24 h and 48 h, respectively (Figure S1C). Hence, these results confirm our hypothesis that a *rhlR* mutant can act as a social cheater in a Δ *lasR* genotypic context.

Identification and isolation of the native prophage of the strain ID4365

Analysis of the ID4365 genomic sequence using the phage predictor program PHASTER revealed the presence of an intact prophage of 58.3 kb in the contig 20 of the ID4365 genome (RefSeq: NZ_ATAI01000086). Detailed annotation of the predicted prophage confirmed the completeness of the structural and replication phage modules, which suggested it was potentially inducible (Supplementary Table 1). To determine whether a prophage in ID4365 was actively induced in our standard growing conditions (BHI and casein media), we spotted filtered overnight culture supernatants in serial dilutions on the bacterial lawns of the strains PA14, PAO1 and PA Φ . Phage plaque formation was observed in the PA Φ lawns, thus corroborating the active prophage induction in strain ID4365. This

same plaque morphology was recognized during the continuous growth experiments of ID4365 in casein from where it was isolated (Figure 1A). This isolated prophage which we named Φ IDHM, was purified and visualized by electron microscopy for its morphological characterization (Ackermann 2007). The viral morphology was an icosahedral head 50 ± 10 nm in length attached to a short tail, typical of a podovirus and usually observed among members of *Podoviridae* (Figure 1B).. We confirmed Φ IDHM resulted from the predicted prophage in the genome of ID4365 by PCR targeted on a specific sequence in the phage, using bacterial genomic DNA and phage DNA from the pure stock as a template. The genomic comparison of Φ IDHM revealed a close relation of this phage with the temperate phages H66 (NC_042342.1) and LKA5 (NC_073675.1), which belong to the F116-Like group (Figure 1C).

Experimental evolution of ID4365 in a continuous growth model in casein leads to population collapses

To investigate cheating with ID4365 in a continuous growth model, we conducted a series of passages to refresh the cultures every 48 h, until the population collapsed. The passages involved cell washing to remove exoproteases and other components in the media, which could influence the growth dynamics (Loarca *et al.* 2019). Four independent cultures of the strain ID4365 in M9 medium with caseinate as the sole carbon source were continuously evolved and consistent with previous studies, the population size decreased with passages (Figure 2A). Population collapses were observed at passage 17 ± 12 , and as we expected, exoprotease-deficient individuals gradually accumulated, reaching for 3 of the 4 cultures approximate maximum levels of 40, 90 and almost 100% prior to the population collapse (at passages 14, 6 and 15, respectively) (Figure 2A and B). Nevertheless, although one of the cultures accumulated similarly high levels of cheaters during different passages, its collapse was prolonged to passage 34, suggesting that other factors besides exoprotease exploitation could be associated with the population collapses.

We hypothesized that one of those factors could be the active production of the Φ IDHM phage during the passages. In agreement with this, the phage titering from growth passages supernatants (normalized by bacterial growth) showed an overall progressive increase during the passages, except for the culture that took longer to collapse, which exhibited more fluctuations for the Φ IDHM induction. However, all cultures displayed a higher phage density prior to the collapses occurring (Figure 2C). For example, phage production of the culture that collapsed earlier (passage 6) increased to $\sim 5.4 \times 10^9$ PFU/mL before the collapse occurred. The cultures that collapsed at passages 14 and 15 had a phage titer of $\sim 10^6$ PFU/mL. Similarly, the culture that collapsed at passage 34 had a phage titer of $\sim 10^7$ PFU/mL. Hence, these results suggested that the accumulation of cheaters and a high prophage induction rate were correlated during the population collapse of the strain ID4365.

To further explore whether the ID4365 cheaters could recover the exoprotease production phenotype by receiving a functional copy of the gene *rhIR*, we transformed two colonies showing low caseinolytic activity and no pyocyanin production isolated from different passages (designated as ID14 and ID16) (Supplementary Table 2) with the vector pGMYC

(pUCP20-*rhlR*⁺). This complementation assay restored exoprotease activity and pyocyanin production for the tested clones. In contrast, the transformation of the clones with the empty vector did not produce any of these effects (Figure 4A and 4B). These results suggested that the clones without these activities potentially were *rhlR* mutants. However, the genome sequencing and mutational scanning analysis of these clones against the parental reference sequence did not show any changes in the gene *rhlR*, its promoter or the whole *rhlA-rarD* operon, which suggest that mutations in other kinds of regulatory genes could also be involved with the loss of the exoproteases activity and pyocyanin production in these clones.

Role of phage ΦIDHM in the growth dynamics and population collapses in PAO1 *lasR* background

The potential role of the temperate phage ΦIDHM triggering population collapses was additionally evaluated using the strain PAO1 *lasR* as a host; this strain was chosen since it is phylogenetically related to ID4365 (Grosso-Becerra *et al.* 2014; Martínez-Carranza *et al.* 2020) and, by having a *lasR* mutant genotype, this strain mimics the condition of the natural host of the phage. The PAO1 *lasR* lysogen was generated using the pure stock of ΦIDHM, isolated from the passages where population collapses occurred. The presence of ΦIDHM as a prophage in the genome of the PAO1 *lasR* lysogen was confirmed by a PCR test that targeted the gene *csrA* present only in the ΦIDHM genome. As expected, the PCR testing produced amplification products for the PAO1 *lasR* ΦIDHM-lysogen and ID4565 DNA but not for the PAO1 *lasR* genomic DNA (Figure S2A). In addition, the PAO1 *lasR* ΦIDHM-lysogen showed resistance to the infection by ΦIDHM, while its isogenic version without the prophage was still susceptible to its infection (Figure S2B).

The PAO1 *lasR* lysogen and its isogenic version without the prophage were grown using the same continuous growth model with caseinate as the only carbon source. Interestingly, the PAO1 lysogen collapsed prematurely at passage 24 ± 2 compared to the no lysogen strain, which collapsed at passage 33 ± 9 (Figure 3A and B) (p-value < 0.05, two-tailed T-test). We also found that the density of the phage ΦIDHM in the lysogen supernatants (normalized by bacterial growth) reached titers around 10^5 and 10^7 before collapsing appeared for most of the cultures (3/4) (Figure 3C), which was similar that we found with the strain ID4365. Furthermore, the percentage of protease-deficient individuals increased during the growth passages for both strains, reaching an accumulation of up to 63 ± 35 for PAO1*lasR* and 47 ± 27 for the ΦIDHM lysogen prior to the collapses (Figure 3D and E). Similarly, as we did for the ID4365 exoprotease cheaters, we selected two non-protease producers colonies derived from the PAO1 *lasR* strain and two from the PAO1 *lasR* ΦIDHM lysogen to complement them with a functional version of the gene *rhlR*⁺, which restored their ability to produce exoprotease and pyocyanin (Figure 4A and 4B). Overall, these results show that the presence of ΦIDHM in a *LasR*⁻ background accelerates the collapse of the population and that the presence of this prophage is associated with a higher vulnerability to social cheating, suggesting a potential role for prophages in a similar context for increasing this detrimental social behavior in natural bacterial populations.

Discussion

Although the utilization of reference strains for the study of ecological interactions such as social cheating is valuable, there could be considerable differences in the regulation of the expression of public goods among environmental and clinical strains. Among these differences is the lack of a functional LasR QS regulator observed in some strains (Cruz *et al.* 2020; Martínez-Carranza *et al.* 2020) and the ability of these strains to still produce significant levels of exoprotease, such as ID4365 and the clinical strain E90, both which utilized RhlR rather than LasR (Cruz *et al.* 2020; Cocotl-Yañez *et al.* 2021). Interestingly, in PAO1 mutant strains with interruptions in *lasR*, some conditions like prolonged starvation select for the restoration of elastase production by increasing the expression of *rhlI* (Van Delden *et al.* 1998), and upon cultivation in low phosphate medium, RhlR regulates the QS response including the production of elastase (Soto-Aceves *et al.* 2020), suggesting that this factor may also orchestrate the QS response in environmental and clinical strains that harbor a functional *lasR* in low phosphate concentrations. To our knowledge, our work is the first report describing exoprotease cheating in a *lasR* natural mutant and it demonstrates that cheating can occur during RhlR-dependent exoprotease production.

In addition, we also further explored the possible role of temperate bacteriophages in exoprotease exploitation dynamics, showing that an active induction of the Φ IDHM prophage in a PAO1 *lasR* mutant accelerated population collapses, increasing the detrimental effects of exoprotease exploitation.

These results are in contrast with our previous findings showing that the presence of the temperate phages D3112 and JBD30 in the PA14 wild-type background aid the wild-type strain in competitions against a QS deficient (*lasR rhlR*) mutant *in vitro* in casein media and *in vivo* in a *Galleria mellonella* infection, counteracting social cheating (Saucedo-Mora *et al.* 2017).

Alternative approaches to evaluate the role of the ID4365 prophage in the cheating dynamics and population collapses could have been to cure the strain from the prophage, or to interrupt or delete genes essential for lysogeny in the ID4365 strain. However, the first option is technically more difficult than making a lysogen with the phage in a reference strain, while the second leaves intact several prophage genes that may play some role in influencing the dynamics and the population collapses. Hence, we think our approach was simpler and adequate. Nevertheless, it should be taken into account that other genes exclusive of either ID4365 or PAO1 *lasR* may also play a role in the observed cheating dynamics.

We hypothesize that a factor that accelerates the population collapses in the strains harboring the Φ IDHM phage, is that their replication during the growth in casein imposes a metabolic burden that perhaps could decrease the production and/or export of exoproteases, since the cellular resources will be used for phage production which tends to increase during the continuous growth passages. Prophage induction also causes the lysis of their host. Figure 5 represents a model describing our findings.

In this work it was not evaluated if the phages can differentially replicate and or lyse the parental or the non-exoprotease-producing mutants; further research is needed to clarify this aspect and determine if this influences the dynamics presented here. Additional ways in which prophages may influence these kinds of dynamics is through their influence in QS, since it is known that several prophages carry QS regulators in their genomes which can modulate the expression of QS controlled traits (Silpe and Bassler 2019; Ambroa *et al.* 2020; Shah *et al.* 2021). Moreover, since some phages can estimate their population size and influence the rate of lytic/lysogenic cycles by a system analogous to QS which is called arbitrium (Erez *et al.* 2017), it would be interesting to determine if those kind of prophages had an effect on cheating dynamics.

The findings shown here encourage further research to unveil yet unknown influences of temperate phages in bacterial cheating of exoprotease dynamics, not only in other clinical or environmental strains but also in reference strains such as PAO1 and PA14 which harbor the filamentous temperate Pf4 and Pf5 phages (Webb, Lau and Kjelleberg 2004; Gavric and Knezevic 2022). Furthermore, a possible role of temperate bacteriophages in the dynamics of the exploitation of other public goods such as siderophores may also exist, hence our work may increase the interest of continue exploring the role of bacteriophages in the sociobiology of their hosts (Secor and Dandekar 2020) and demonstrate that the study of non-canonical reference strains enriches our knowledge of the bacterial physiology and ecology.

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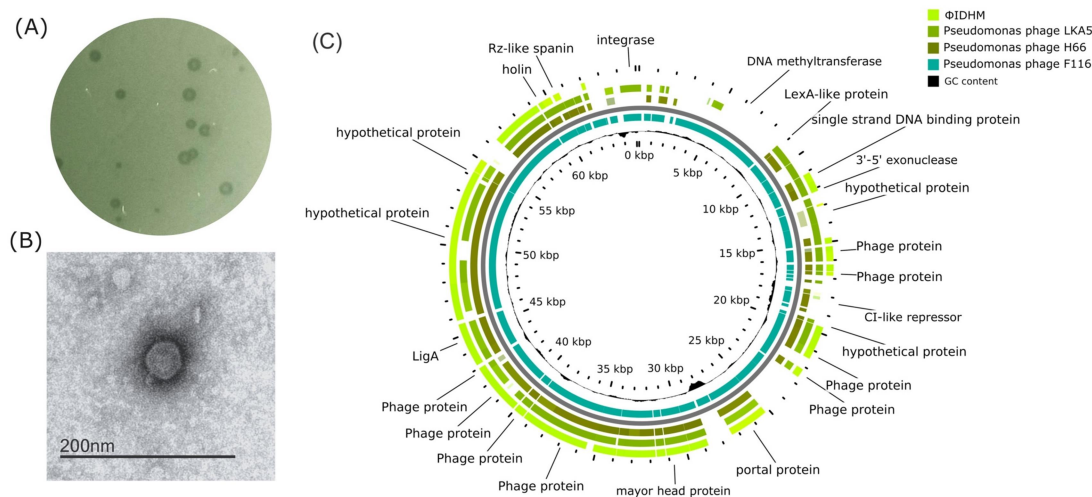


Figure 1. Phenotypic and genotypic properties of temperate phage Φ IDHM isolated from the environmental strain ID4365. A) Plaque morphology of Φ IDHM in a bacterial lawn of PA Φ . B) Electron micrograph of a Φ IDHM's virion, exhibiting the typical morphology of a podovirus. C) Genomic circular comparison of Φ IDHM with three of its homologous from the F116-Like phage group.

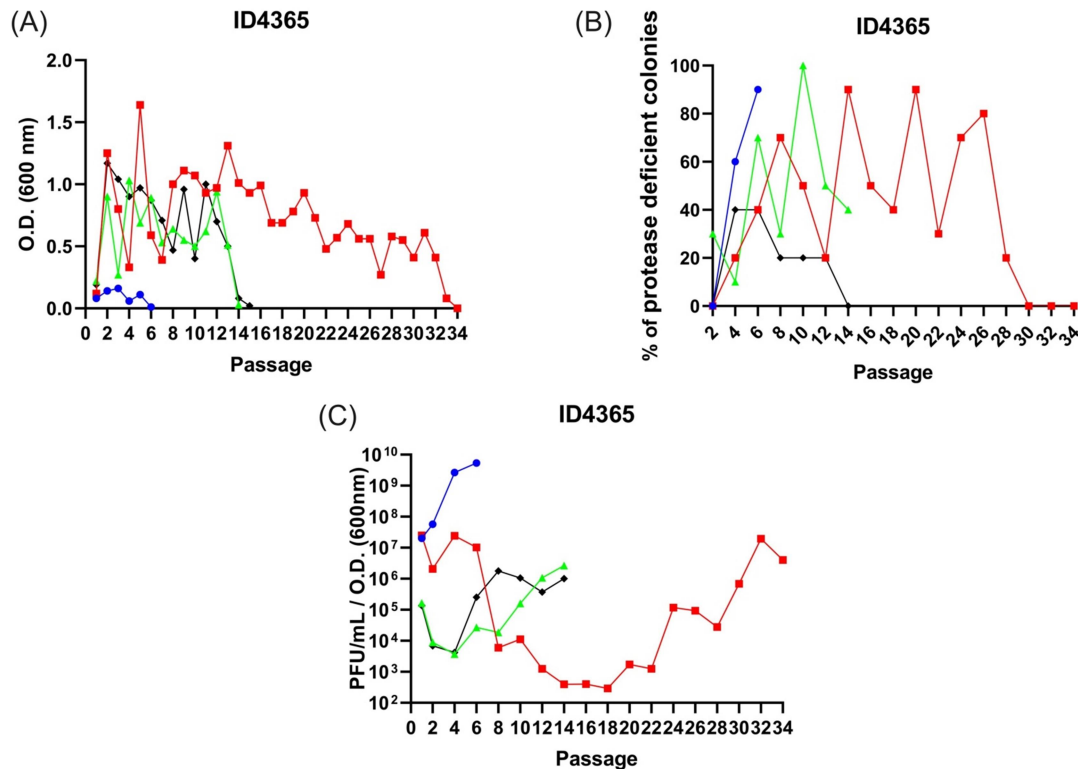


Figure 2 Growth dynamics of the ID4365 strain in M9 medium with 0.25% caseinate as sole carbon source. The first passage was inoculated using overnight cultures from single ID4365 colonies grown in BHI medium and subsequent growth passages were done every 48 h and inoculated with bacteria from the previous passage to an initial turbidity of 0.05 (O.D. 600 nm). Results for 4 independent experiments are shown in different colors. A) ID4365's population density estimated by OD reads (600 nm) during the growth passages. B) Percentage of protease-less colonies obtained from each growth passage of the experiments shown in Figure 1A. C) Φ IDHM phage titers from even growth passages of the cultures shown in Figure 1A, titers were normalized by the corresponding bacterial growth.

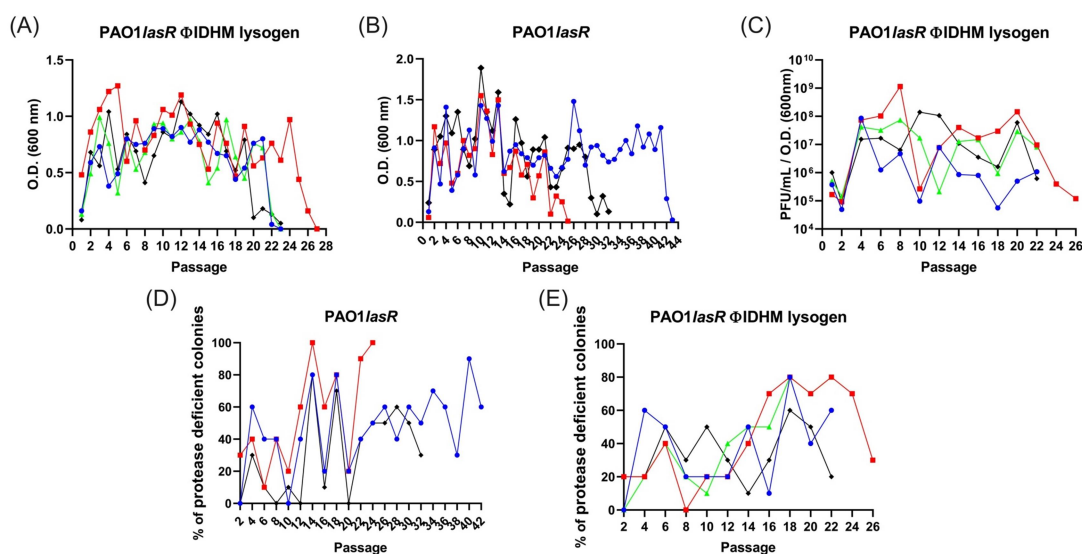


Figure 3 A) and B) Continuous growth of PAO1, *lasR* ΦIDHM lysogen, and PAO1 *lasR* strains in M9 medium with 0.25% caseinate as sole carbon source. Results for independent experiments are shown in different colors.

C) ΦIDHM phage titers from even growth passages of the cultures shown in figure 2A for the PAO1 *lasR* ΦIDHM lysogen strain, titers were normalized by the corresponding bacterial growth (O.D. 600 nm).

D) and E) Percentage of protease-less colonies obtained from even growth passages of the experiments shown in figure 2A and 2B, respectively.

ORIGINAL UNEDITED MANUSCRIPT

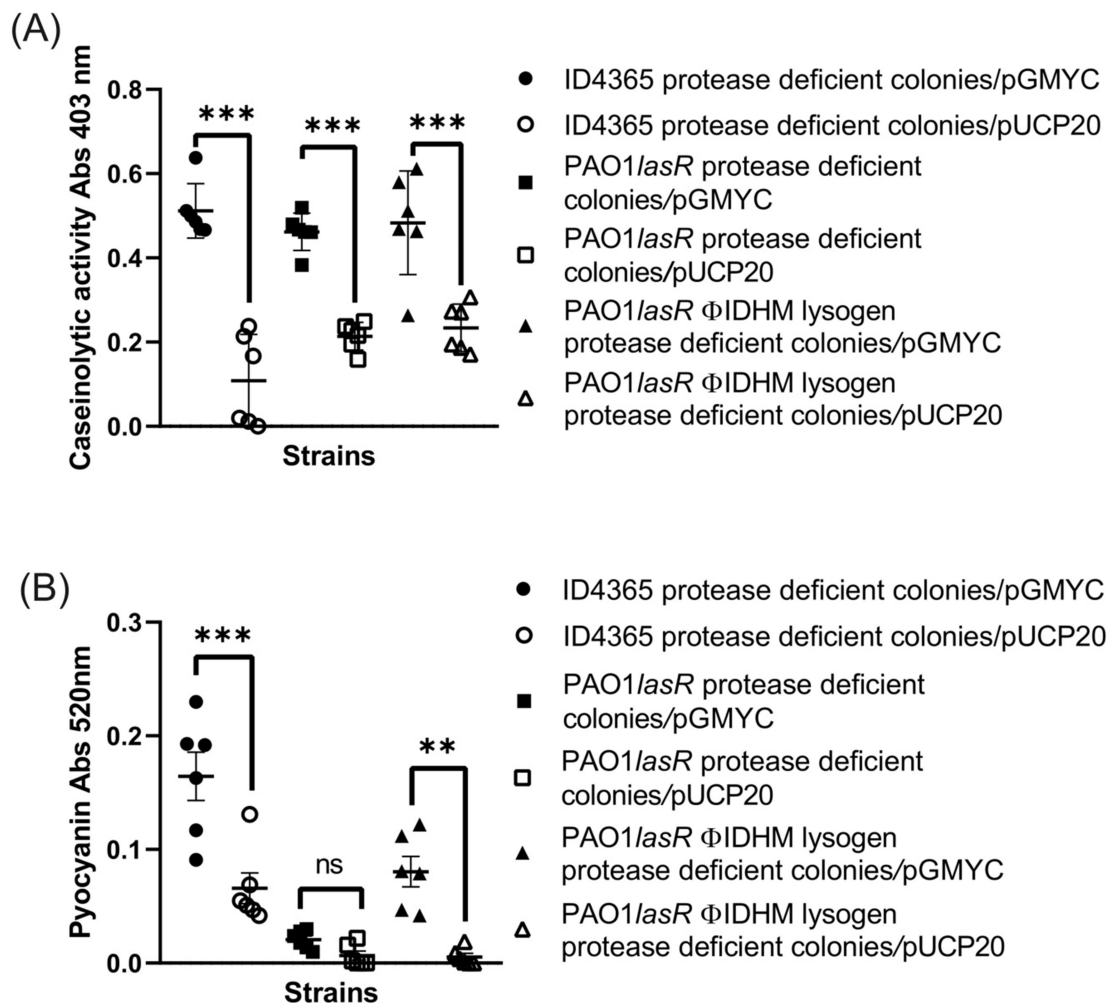


Figure 4 A) Caseinolytic activity and B) pyocyanin production of two ID4365 exoprotease-deficient clones transformed either with pUCP20 or pGMYC, two PAO1 *lasR* exoprotease-deficient clones transformed either with pUCP20 or pGMYC and of two PAO1 *lasR* ΦIDHM lysogen exoprotease-deficient clones transformed either with pUCP20 or pGMYC.

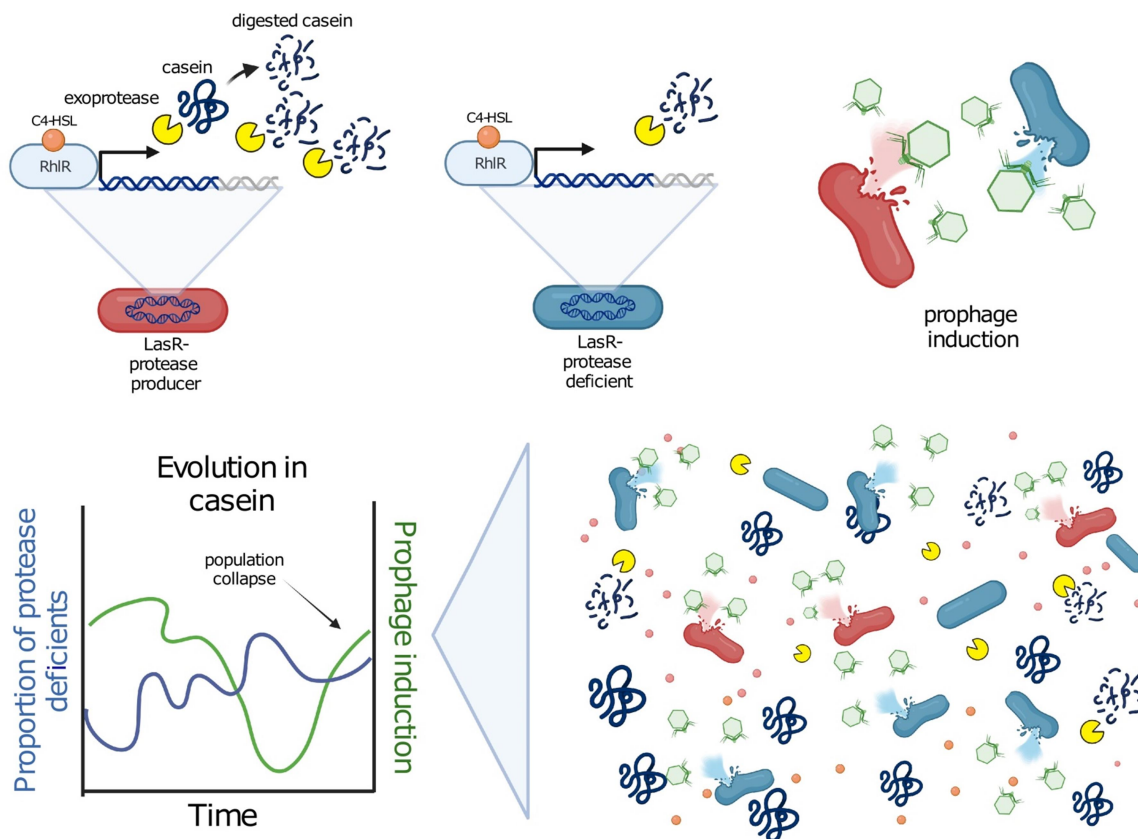


Figure 5) Graphical abstract of showing the interactions between LasR⁻ protease producers and LasR⁻ protease-deficient individuals with casein as the sole carbon source and showing that prophage induction in both subpopulations promotes population collapse. Created with BioRender.com