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SHORT COMMUNICATION

High local substrate availability stabilizes a cooperative trait

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Cooperative behavior is widely spread in microbial populations. An example is the expression of an extracellular protease by the lactic acid bacterium *Lactococcus lactis*, which degrades milk proteins into free utilizable peptides that are essential to allow growth to high cell densities in milk. Cheating, protease-negative strains can invade the population and drive the protease-positive strain to extinction. By using multiple experimental approaches, as well as modeling population dynamics, we demonstrate that the persistence of the proteolytic trait is determined by the fraction of the generated peptides that can be captured by the cell before diffusing away from it. The mechanism described is likely to be relevant for the evolutionary stability of many extracellular substrate-degrading enzymes.

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Evolutionary game theory has been applied in a number of recent studies to explain the dynamics of microbial populations (Kerr *et al.*, 2002; Griffin *et al.*, 2004; Kirkup and Riley, 2004; MacLean and Gudelj, 2006; Gore *et al.*, 2009). Here, we show that it can be applied also to predict the dynamics of mixed populations of protease-positive and -negative strains of the lactic acid bacterium *Lactococcus lactis. L. lactis* is auxotrophic for a number of amino acids. To grow in one of its preferred niches, milk, it is dependent on a protease that degrades milk proteins into utilizable peptides (Kunji *et al.*, 1996). This protease is anchored to the cell wall, and the peptides resulting from the cleavage of milk proteins can be taken up or diffuse away from the cell (Supplementary Figures S1 and S2). Cheating, protease-negative (prt^-) strains can use the peptides generated by the cooperative protease-positive (prt^+) strains without having the burden of protease expression. This allows prt^- individuals to invade a population of prt^+ individuals (Hugenholtz *et al.*, 1987). The extracellular protease is often plasmid encoded, which upon loss leads to the rapid appearance of prt^- mutants in a prt^+ population (McKay and Baldwin, 1975). In serial propagation experiments, the fraction of prt^+ strains in a mixed culture will decrease, leading to a decreased overall growth rate (Supplementary Figure S3), as well as to a decreased biomass yield (Supplementary Figure S4). As a result, a rapidly growing prt^+ culture can turn into a poorly growing prt^- culture when propagated in milk. This phenomenon, first described in 1931 (Harriman and Hammer, 1931), has troubled the dairy starter industry for a long time, and has strongly influenced practices for starter culture and cheese production.

The instability of the cooperative prt^+ phenotype is clearly determined by the extracellular character of the protease. We reasoned that the existence of high local peptide concentrations, generated by the extracellular substrate-degrading enzyme, could play a key role in the evolution and stable coexistence of the two variant strains. This mechanism would provide an advantage to prt^+ cells over prt^- cells, particularly at low cell densities and concomitantly low global peptide concentrations. At high cell densities, the prt^+ strain has the burden of protease expression, whereas the prt^- cheater can thrive on relatively high free peptide concentrations without having the extra burden. A similar mechanism, dependent on nutrient gradients created around the cell, was suggested for the evolutionary

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stabilization of invertase expression in *Saccharomyces cerevisiae* (Greig and Travisano, 2004; Gore *et al*., 2009). Despite the plausibility of the existence of such localized substrate gradients, the experimental evidence presented remained indirect.

To directly measure the proposed localized substrate availability around prt$^+$ cells, we transcriptionally fused the bacterial luciferase reporter genes (*luxAB*) to the lactococcal promoters of the *metE* and *dppA* genes (Bachmann *et al*., 2010) that respond to intracellular peptide and amino-acid levels (den Hengst *et al*., 2005), whereby luciferase activity acts as an intracellular amino-acid and peptide sensor (Supplementary Table 1). The *luxAB* reporter constructs were transformed into *L. lactis* MG1363, a prt$^-$ strain, and MG610, a derivative of MG1363, which contains two copies of the protease gene *prtMP* stably integrated into the genome (Leenhouts *et al*., 1991). Subsequently, it was established in pure cultures that expression of both reporter constructs is downregulated when residing in the prt$^+$ strain as compared with the prt$^-$ strain (Supplementary Figure S5). Mixed cultures with different prt$^+$/prt$^-$ ratios and containing the reporters for intracellular amino-acid availability in either of the two host strains were studied (Supplementary Figure S1 and Supplementary Table 2). If the proposed model of a host-dependent localized peptide availability is correct, a cell density-dependent and host-specific response of the peptide and amino-acid sensor system is expected throughout the growth of a batch culture. This host-specific response can be measured as the slope of a sliding window for each consecutive measurement of the luminescence time series. In cultures with a relatively high proportion of prt$^+$ cells (90% and 50% prt$^+$), the luminescence signals differed very little, irrespective of whether the reporter was in the prt$^+$ or the prt$^-$ strain (Figures 1a–d). This indicated that in these mixed cultures, the level of extracellular protease activity is apparently sufficient to generate peptide levels that allow repression of the *dppA* and *metE* promoters, also in the prt$^-$ strain of a co-culture. In contrast, when the culture contained only 10% prt$^+$ cells, a distinct difference was measured between the *dppA*- (Figures 1e and f) or *metE*- (Supplementary Figure S6) sensor activity in the prt$^+$ and prt$^-$ strains. Initially, a rapid increase of the luciferase signal, coinciding with the depletion of the free amino acids and peptides in milk, is observed in both strains. However, the subsequent phase is characterized by a steady increase of the luminescence signal when the reporter resides in the prt$^-$ strain, whereas a decreasing relative expression per cell is observed for the reporter residing in the prt$^+$ strain. The high *dppA*- and *metE*-sensor activity in the prt$^-$ relative to the prt$^+$ host indicates that the level of intracellular peptides is lower in the prt$^-$ host. Because these measurements were made in mixed cultures, they support the proposed high local peptide availability for the prt$^+$ host. DppA expression is controlled by the

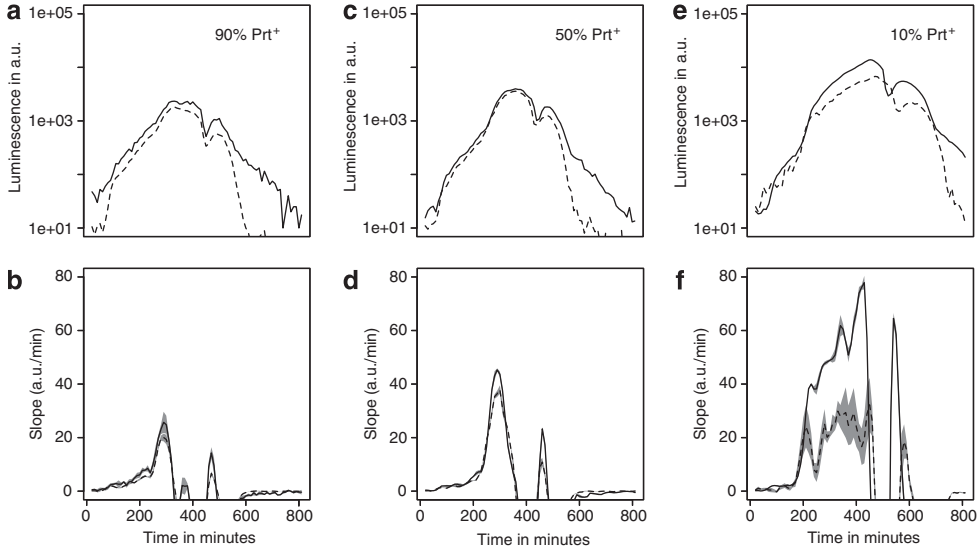

Figure 1 Localized peptide concentrations measured by intracellular luciferase-based peptide sensing. Mixed batch cultures of prt$^+$ and prt$^-$ strains were grown in reconstituted skimmed milk. The fraction of the prt$^+$ strains in the co-culture were 90% (**a**, **b**), 50% (**c**, **d**) and 10% (**e**, **f**). Intracellular peptide concentrations were measured via the *dppA*-controlled luminescence signal and are given in arbitrary units (*y* axis, top panel). The *dppA* reporter construct resides either in the prt$^+$ (dashed line) or the prt$^-$ (solid line) host strain. The presented data is corrected for the relative abundance of the strain carrying the luciferase reporter. The slopes of the luminescence traces are given on the *y* axis of the lower panels. Each curve represents the average of four biological replicates. The results show that at a high relative abundance of the prt$^+$ strain, the *dppA* expression levels are similar, indicating little or no difference in peptide availability for the two strains (**a–d**). At low frequencies of the prt$^+$ strain, the intracellular amino-acid levels are higher in the prt$^+$ strains (**e**, **f**), which is detected by the downregulation of *dppA* expression in that strain. For the lower panels, the standard error is shaded in gray. The dip in luminescence activity between 400 and 500 min on the *x* axis is an intrinsic property of the luminescence reporter, which is linked to changes in metabolic activity when cells go into stationary phase (Bachmann *et al*., 2007).

pleiotropic transcriptional repressor CodY, which is activated at low concentrations of branched chain amino acids and described to have over 100 potential targets (Guedon *et al.*, 2005). The presented data therefore suggests that the complete CodY regulon might be differentially regulated in the prt$^+$ cells as compared with the prt$^-$ cells. It should be noted that *L. lactis* is a non-motile bacterium and that all measurements were made in static cultures, leaving diffusion as the determining factor of peptide movement (Supplementary Figure S2).

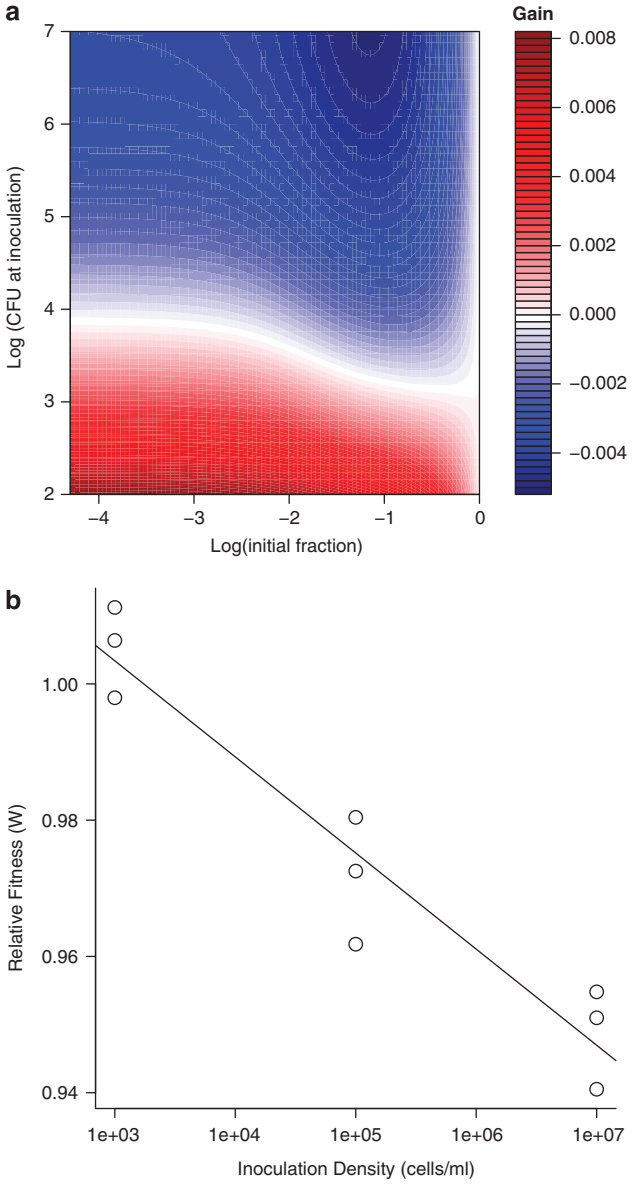

Figure 2 (**a**) Modeling population dynamics of prt$^+$ and prt$^-$ mixed strain cultures. The heat map displays the fractional gain of the prt$^+$ strain after one culturing step. The dependency of this fractional gain on the initial fraction of the prt$^+$ cells in the culture (*x* axis) and the inoculation density in colony forming units (CFU) (*y* axis) is shown. With increasing inoculation densities and/or an increasing fraction of the prt$^+$ strains in the culture, the overall advantage of the prt$^+$ strain vanishes. Model and parameters are given in the supplementary information. (**b**) Relative fitness (*y* axis) of prt$^+$ strains if propagated at different cell densities. Equal amounts of prt$^+$ and prt$^-$ strains were inoculated in milk, and propagated for about 100 generations. The inoculation densities at each propagation event are indicated (*x* axis). For each condition, three biological replicates were propagated (o). Linear regression shows a highly significant correlation between the fitness of prt$^+$ strains and the inoculation density ($R^2 = 0.91$, $P < 0.0001$). The prt$^+$ strain could be stabilized in the culture when propagated at low cell densities ($W \sim 1$) and their abundance in the culture decreased when propagated at high cell densities ($W < 1$).

Population dynamics of the two competitors were described in a mathematical model, taking into account the influence of cell density and population composition on the growth rates of the two host strains in a mixed culture (Figure 2a and Supplementary Information). Model predictions were confirmed by serial dilution experiments of cultures propagated at different inoculation densities. The relative fitness of the prt$^+$ strain was significantly increased when cells were propagated at low densities (Figure 2b). Although it would be desirable to observe the invasion of the prt$^+$ strains from low frequencies in cultures propagated at low inoculation densities, such an analysis is complicated by the fact that wild-type protease carrying plasmids also harbor other functions such as peptide transporters (Siezen *et al.*, 2005). The fact that the extracellular protease is usually plasmid encoded in lactococci is of interest in the light of a recent analysis, showing that cooperative traits are overrepresented on mobile genetic elements, and suggesting that horizontal gene transfer promotes cooperation (Nogueira *et al.*, 2009).

A recent study in yeast (Gore *et al.*, 2009) demonstrated snowdrift game population dynamics, known from game theory. In contrast to the prisoners dilemma, where cooperators are not maintained, snowdrift game dynamics allow the maintenance of cooperators at non-zero frequencies (Doebeli and Hauert, 2005). The same snowdrift game dynamics is likely to apply for the proteolytic trait of lactococci. The appearance of cheating prt$^-$ lactococci is frequently observed, and has been investigated in great detail based on its importance for starter culture performance in the dairy industry (McKay and Baldwin, 1975; Otto *et al.*, 1982; Hugenholtz *et al.*, 1987). The maintenance of biodiversity is influenced by spatial structure (Chesson, 2000), which has been demonstrated earlier for microbial systems (Rainey and Travisano, 1998). Our example shows direct evidence of localized substrate concentrations in suspensions cultures. The demonstration that such localized substrate availability affects intracellular regulatory mechanisms has also direct implications on studies of culture heterogeneity, far beyond those of substrate-degrading enzymes.

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