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Microbes are not bound by sociobiology: Response to Kümmerli and Ross-Gillespie (2013)

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In recent years, sociobiology has been extended to microorganisms. Viewed through this lens, the microbial world is replete with cooperative behaviors. However, little attention has been paid to alternate hypotheses, making many studies self-confirming. Somewhat apart is a recent analysis of pyoverdin production—a paradigmatic public good and social trait—by *Pseudomonas*, which has revealed discord between predictions arising from sociobiology and the biology of microbes. This led the authors, Zhang and Rainey (Z&R), to question the *generality* of the conclusion that pyoverdin is a social trait, and to question the fit between the sociobiology framework and microbiology. This has unsettled Kümmerli and Ross-Gillespie (K&R), who in a recent "Technical Comment" assert that arguments presented by Z&R are flawed, their experiments technically mistaken, and their understanding of social evolution theory naive. We demonstrate these claims to be without substance and show the conclusions of K&R to be based on a lack of understanding of redox chemistry and on misinterpretation of data. We also point to evidence of cherry-picking and raise the possibility of confirmation bias. Finally, we emphasize that the sociobiology framework applied to microbes is a hypothesis that requires rigorous and careful appraisal.

KEY WORDS: **Alternate hypothesis, falsifiable data, public good, siderophore, social evolution.**

Biologists strive to make sense of a rich and diverse natural world. Observation fuels ideas, which drive empirical analyses, leading to hypotheses—often informed by abstract mathematical models—and further experimentation. On occasion, theory with general explanatory power emerges (Popper 1959; Kuhn 1962). Application of theory aids further discovery. Because theory shapes experimental expectation, it is important that theory aligns with the biological problem under investigation. Ensuring appropriate alignment depends on objective and critical appraisal of alternate hypotheses.

Over the last decade the idea that the microbial world is inherently social has moved from interesting hypothesis to seemingly established fact (see Zhang and Rainey 2013 [hereafter Z&R] and references therein). However, with a few notable exceptions (see Z&R), a careful appraisal of the literature would leave an objective reader less than convinced. Claims are based largely on the

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discovery—and for the most part in vitro generation—of mutants that do not produce extracellular products. Those advocating a social perspective label nonproducers "social cheats" (types that gain advantage from producing types) and thus it follows (according to the social evolution perspective) that producing types are "cooperators"; any secreted product is termed a "public good" (West et al. 2006, 2007a; Nadell et al. 2009; Foster 2010). Although this may be true, there exist alternate nonsocial explanations for producers and nonproducers (elaborated in Z&R).

Experiments to test whether the social evolution hypothesis appropriately describes the microbial world are possible. Indeed, where secreted products are public goods in a strict game theoretic sense,[1] for example, where the product is equally available to producers as to nonproducers (Olson 1965; Hardin 1968; Dionisio and Gordo 2006), theory makes a number of simple predictions: nonproducers will gain advantage in the presence of the secreted product; nonproducers will do poorly in the absence of producers; producers will do better in the absence of nonproducers (West et al. 2006, 2007a). If evidence arises consistent with the public good hypothesis, then further experiments allow the hypothesis of cooperation (as adaptation) to be tested. Although it is often assumed that evidence consistent with the public goods hypothesis establishes the underlying trait as cooperative, this need not be true (Driscoll et al. 2013). To claim that a given product is cooperative is to imply that its origin (and/or maintenance) is at least partly attributable to selection acting on some beneficial effect conferred on recipients. It is thus necessary to show that production of the public good incurs a cost and that production has been selected (or is maintained) because of some benefit conferred on recipient cells (West et al. 2007b). It is also important to establish the nature of the presumed collective benefit. When it comes to microbes this is no trivial matter: it requires knowledge of the ecological circumstances under which a given trait evolved (or is maintained).[2]

Experiments to test the public good hypothesis are in principle straightforward, requiring simple growth experiments. However, the outcome of such experiments depends on choices made by investigators. Significant factors include the nature of the test environment (e.g., culture medium and conditions of culture), founding density of bacteria, ratio of competitors, preculture conditions, and specific details of competing genotypes. Of these, the most critical is the nature of the test environment.

The appropriate environment is one in which the presumed social trait evolved or is maintained. If the focus of interest were cooperative hunting in Serengeti lions, then the Serengeti Plains would be the focus of study. For microbes, knowledge of the appropriate environment is rarely available; rarely is the ecological significance of presumed social traits understood to the necessary degree; rarely is there sufficient understanding of the ecophysiology of the trait of interest, or knowledge of the ecological circumstances under which the trait evolved and/or is maintained. Although the value of performing studies of cooperative hunting in lions, using caged lions and captive gazelles, would be questionable, studies of social interactions in microbes are unhesitatingly performed under conditions likely to fall well short of approximating appropriate ecological conditions. Where the goal is to construct a biological model, this can be justified; however, when researchers reach general conclusions regarding the nature of interactions informed only by laboratory experiments, then choice of environment is of paramount importance. Here there is need for care: the experimenter has god-like power. It is a simple matter to inadvertently contrive laboratory conditions so as to ensure producers and nonproducers perform in accordance with preconceived notions.

Pyoverdin, Paradigmatic Public Good?

The water-soluble iron-chelating compound pyoverdin, produced by members of the genus *Pseudomonas* (Stanier et al. 1966), has become the exemplar of "social trait" and "public good" (see Z&R). The yellow-green fluorescent pigment has long held diagnostic value for clinical microbiologists. Studies of the pigment and conditions promoting its production date back to the 1890s (Gessard 1892; Georgia and Poe 1932). These early studies showed that pyoverdin production is stimulated by growth in media low in iron (Paton 1959), but too little iron interferes with growth (Garibaldi 1967). Since 1954, the preferred (proteose peptone-based) medium for culture of *Pseudomonas* (and enhancement of pyoverdin production) has been King's Medium B (KB) (King et al. 1954).[3]

Pioneering work performed more than 35 years ago showed that pyoverdin is a desferrisiderophore (Meyer and Abdallah 1978): it also has additional roles, for example, it appears to

[1]Not all cooperative traits are public goods in this strict sense, but such a definition has value because it generates readily testable predictions (see West et al. 2006, 2007a). A key issue for future investigation is the degree to which a given product is apportioned between a given producing cell and any neighboring cells.

[2]We note, but question, K&R's watered-down definition of a cooperative trait. K&R state "cooperative traits are defined as behaviours that have positive fitness consequences for others." K&R cite West et al. (2007b), but these authors go to some length to explain why cooperation poses a dilemma for evolution and why, for example, elephant dung is not a cooperative trait even though defecation benefits dung beetles. According to K&R's definition, elephant defecation qualifies as a cooperative trait. It is unclear what this overly broad definition might contribute to the challenge of explaining cooperation, except perhaps to shift the burden of proof upon the increasingly neglected possibility that a trait might not be social.

[3]The paper by King et al. (1954) has been cited in excess of 4000 times.

function as a heavy metal tolerance and resistance system (Braud et al. 2010; Hannauer et al. 2011; Schalk et al. 2011). Numerous subsequent investigations have revealed much regarding the genetics and biochemistry of pyoverdin production (reviewed in Visca et al. 2007), but many aspects of pyoverdin ecophysiology remain uncertain (Kraemer 2004).

Dozens of papers published in the last decade assert that pyoverdin is a costly public good and cooperative (even altruistic) trait (see references in Z&R). Often cited evidence stems from experiments performed by Griffin et al. (2004).[4] Given the importance of this article, a close look is warranted. The authors monitored growth of a pyoverdin-producing and nonproducing strain of *P. aeruginosa* PAO1 in casamino acids medium (CAA) and in CAA supplemented with varying levels of an iron-chelating agent (apotransferrin), in both monoculture and mixed culture. Statistical analyses showed that in CAA with high levels of apotransferrin (but not in CAA where pyoverdin is nonetheless produced) nonproducers gained advantage from producing strains and producers reach higher densities in monoculture than nonproducers. Griffin et al. (2004) also reported the results of analyses showing a cost to pyoverdin production in iron-replete media,[5] but this remains difficult to understand in light of known iron-mediated repression of pyoverdin synthesis (Meyer and Abdallah 1978; Ochsner et al. 1995; Hassett et al. 1996) and could not be verified by Z&R. Although Griffin et al. claim their data provides evidence that pyoverdin production is a "costly altruistic trait," a less generous reader might conclude the data to be consistent with the hypothesis of pyoverdin as a public good (under a limited set of conditions where pyoverdin behaves as a public good), but falls short of establishing pyoverdin as cooperative, or altruistic trait. A critical reader might point to the fact that the hypothesis that pyoverdin is a public good is unlikely to be rejected when the chosen environment (CAA supplemented with apotransferrin) ensures that producers and nonproducers interact in accord with this framework. Further concerns stem from the fact that the nonproducing type did not evolve in any of the focal environments, but was the product of uncharacterized in vitro mutagenesis (Hohnadel and Meyer 1988).

Although we readily acknowledge the value of Griffin et al. (2004)—especially with regard to insights into the scale of competition—subsequent studies have delivered little additional data to strengthen the hypothesis that pyoverdin is a social trait (see Z&R for references). In our opinion, such studies, and those of other extracellular microbial products (now also considered social traits), typically overlook the critical difference between demonstrating competitive dynamics consistent with a public goods problem (not necessarily to the exclusion of other explanations), and conclusive demonstration that a trait has evolved and/or is maintained as a social adaptation. Invoking cooperation (where there exists a cost to the actor) or altruism as the cause of a particular trait is necessarily an adaptive hypothesis, which bears on evolutionary history, whereas the public goods game describes interactions among co-occurring populations. Just why observations consistent with public goods dynamics have been taken as conclusive proof of evolved altruism is not clear, but it may be in part a consequence of the asymmetric demands of studying evolution in macroscopic versus microscopic organisms.

Many decades ago, evolutionary biologists observed indisputable examples of evolved altruism (e.g., sterile worker castes), and were challenged to develop frameworks capable of explaining such observations. Modern socio-microbiologists face the opposite challenge: theoretical frameworks for explaining social adaptation are well developed, but identifying adaptations in profoundly unfamiliar contexts, and in organisms that cannot be observed with the naked eye, is a difficult task. The public goods dynamics that may adequately describe interactions between producing and nonproducing strains in laboratory cocultures bear on the forces driving evolution of the underlying traits only insofar as laboratory conditions reproduce salient features of the environment in which the adaptation arose, or is maintained.

The Study of Zhang and Rainey (2013)

In a recent paper, Z&R reported the results of experiments aimed at testing the hypothesis that pyoverdin produced by *P. fluorescens* SBW25 is a public good. In these experiments, the authors demonstrate that pyoverdin producers and nonproducers behave in accord with predictions from social evolution theory, but only under a limited set of environmental (and genetic) conditions. Central to their study was a 600-generation selection experiment, performed in KB—in structured and unstructured microcosms—in which nonproducing types evolved de novo. These types were genetically characterized, an isogenic strain constructed, and its performance subsequently studied in structured and unstructured microcosms, and at different founding ratios. Ensuing data led to rejection of the hypothesis that *nonproducers evolving in KB* gain advantage by taking unfairly of pyoverdin from producing cells. In KB, nonproducers arise by mutation in the regulator gene *pvdS* and gain advantage by avoiding costs associated with a regulatory system that is maladapted to life in planktonic laboratory culture.

[4]West and Buckling (2003) is also often cited, but this article contains no empirical data.

[5]The nature of this environment is unclear. Griffin et al. (2004) do not mention supplementation with iron, but nonetheless they claim iron-replete conditions. The reader thus assumes the iron-replete environment to be unsupplemented CAA, but CAA promotes production of pyoverdin due to its iron-deplete status.

Extensive additional data led Z&R to question the *generality* of the claim that pyoverdin is a public good, producers are cooperators, and nonproducers are social cheats. Their findings also led them to question the appropriateness of the fit between the social evolution framework and the lives of microbes. At no point did Z&R reject outright the hypothesis that pyoverdin is a public good or a cooperative trait. To quote:

> Our work shows that under certain laboratory conditions (media supplemented with high levels of iron-chelating agent) pyoverdin behaves as expected of a public good, however conformity to the social evolution framework is dependent on both genotype and environment. In some environments, pyoverdin-defective types evolve because production of pyoverdin is maladaptive. Under other conditions pyoverdin appears to be personalized. The discovery of just one set of conditions under which pyoverdin is produced, and yet does not behave in accord with social evolution theory, gives reason to question the generality of the conclusion that pyoverdin is a public good, that producers are cooperators and that non-producers are social cheats.

These findings and ensuing conclusions have unsettled Kümmerli and Ross-Gillespie (hereafter K&R). In a recent comment, K&R call into question the experiments and conclusions put forward by Z&R, claiming their work to be flawed and their understanding of social evolution naive. We reject such assertions. In the following, we show that the work of Z&R is valid, and present empirical evidence and logical arguments that counter the charges of K&R.

Technical Sufficiency With The Growth Medium

It is a serious allegation to assert that a published work is flawed. If correct, it warrants retraction of the offending article. Naturally one assumes that those making the allegation have incontrovertible evidence. Logically, such evidence stems from replication of the alleged flawed experiment, alongside a treatment in which the flaw and its effects are put right, thus highlighting the flaw. K&R repeat no part of Z&R's study and present no defensible case for the claim that the work of Z&R is technically insufficient, or based on flawed argument.

In the opinion of K&R, Z&R used the wrong medium[6] and are inconsistent in their use of iron chelators.[7] KB, according to K&R, should not be used to study the "social evolution" of pyoverdin because it is replete with iron and promotes the production of negligible amounts of the siderophore. K&R present data to back these two central criticisms, but upon further investigation (and in light of the data K&R provide), we find serious problems with each line of argument. We first call attention to basic principles of iron biochemistry that, when properly appreciated, invalidate their first claim. Their second claim stands in the face of more than 60 years use of KB to culture and identify *Pseudomonas*—precisely because it does promote pyoverdin production. But more significantly, the claim that KB does not promote pyoverdin production is dependent on presentation of data in a manner that ignores well-known density-dependent effects. When such effects are recognized (and the raw data of K&R examined), it becomes apparent that K&R's own data demonstrate production of pyoverdin in KB at levels no different to those observed in CAA.

MEASURES OF TOTAL IRON ARE NOT INDICATIVE OF THE IRON THAT IS AVAILABLE TO MICROBIAL LIFE

KB is a nutritionally complex medium, containing, in addition to salts, 20 g L^{-1} proteose peptone and 10 g L^{-1} glycerol. CAA, on the other hand, is a nutritionally compromised medium: in addition to salts, CAA contains just 5 g L^{-1} casamino acids[8] and no additional carbon source. K&R use a commercial "QuantiChrom" iron assay kit to measure total iron[9] in KB and CAA. They show KB to have higher total iron than CAA. The finding that KB has more total iron than CAA is unsurprising, but moreover, it is irrelevant. What is relevant is the chemical form of iron. This determines solubility and thus availability of iron for microbial growth.

Inorganic chemistry provides an accessible explanation for why iron availability depends primarily on the state of iron, rather than its absolute abundance (Lippard and Berg 1994). Iron exists in more than one oxidation state—the two common states

[6]Although K&R insist that CAA supplemented with 100 μg mL^{-1} apotransferrin is the correct medium, many studies on the sociobiology of pyoverdin use KB. Moreover, Z&R performed an extensive set of experiments in CAA and show that provided the environment is structured and the genotype of the nonproducer carries a *pvdS* mutation (and not a deletion of *pvdL*), then pyoverdin behaves as expected of a public good.

[7]Z&R are remarkably consistent in their use of iron chelators—the study of Z&R is unique in having identified nonproducing mutants arising de novo, having genetically characterized these mutants, and having investigated their interaction with producing types in the very same environment in which they evolved. This is in contrast to the use of genetic mutants that may have no ecological or evolutionary relevance in the chosen test environment.

[8]Casamino acids are a mixture of amino acids (but lacking cystine and tryptophan and largely deplete in vitamins) derived from acid hydrolysis of casein. They are traditionally used as a supplement to minimal growth media. CAA contains 5 g L^{-1} casamino acids (plus salts). It is a nitrogen-rich, carbon-limited medium that promotes poor microbial growth. Its formulation and infrequent use in the study of pyoverdin stemmed from the need for a low salt medium for biochemical analyses of the molecule involving isoelectric focusing.

[9]K&R refer to the iron assayed as soluble iron, but according to the QuantiChrom assay kit, the product measures total iron following addition of a reducing agent to convert insoluble ferric iron to the soluble ferrous form.

are the reduced ferrous (Fe(II)) and oxidized ferric (Fe(III)) forms. Oxidation state matters, because it dramatically affects solubility and thus availability of iron to life. In aerobic environments at neutral pH iron exists in the ferric state as oxyhydroxy polymers and is highly insoluble ($K_s \sim 10^{-38}$ M), and thus unavailable (Hallberg 1981; Neilands 1981). Many additional and complex factors also affect availability, such as the presence of ligands—including natural organic ligands, for instance certain amino acids and peptides—which are features of both KB and CAA. Any comment on the iron status of KB versus CAA must consider these complexities; moreover, to have biological relevance any measurement of iron content must distinguish available iron from unavailable iron—a tall order that requires sophisticated voltametric methods (e.g., Rue and Bruland 1995) or bioassay (e.g., Loper and Lindow 1994). K&R do not accomplish this. The QuantiChrom iron assay kit, which measures total iron via an undisclosed proprietary process,[10] is insufficient.

K&R'S OWN DATA DEMONSTRATE THAT KB PROMOTES PRODUCTION OF PYOVERDIN

It is not necessary to resort to a chemical assay to determine whether KB is low in available iron. Such a claim can be made based on the behavior of the bacterium itself: the bacterium is a biosensor (Joyner and Lindow 2000; Chiadò et al. 2013). Pyoverdin is produced as a direct response to low levels of available iron (e.g., Meyer and Abdallah 1978): addition of surplus iron suppresses pyoverdin production (e.g., Meyer and Abdallah 1978).[11] Z&R report having performed such an experiment. We further replicate it here. However, before doing so, the data of K&R warrant inspection.[12]

Column five of Table S2 (see Table S2 of K&R) reports optical density (OD) (as a rough proxy for cell number) determined after 24 h growth in KB and two variants of CAA. Column six reports relative fluorescence units of pyoverdin (RFU) determined at the same time using a spectrophotometer (as a proxy for pyoverdin concentration). With attention on data from wild-type *P. aeruginosa*, K&R report OD_{600} measures that range from ~0.03 (CAA [BD]) to ~1.3 (KB),[13] and measures of pyoverdin that are as follows: for KB, 12,326 RFU (11,653–12,999); for CAA (BD), 11,127 RFU (9744–12,510); and for CAA (Sigma), 12,442 RFU (11,638–13,245) (mean RFU [and 95% confidence intervals] from six replicates). The data show that while cell density varies dramatically depending on the medium, there is no significant effect of medium on total amount of pyoverdin: K&R's data demonstrate that KB promotes production of pyoverdin.

The claim that KB promotes only minimal production of pyoverdin depends entirely on expression of pyoverdin as a function of OD (RFU/OD_{600}). Given the superior nutritional status of KB relative to CAA, cells in KB grow to a much higher density than in CAA. The significantly greater denominator means that KB-grown cells *appear* to produce considerably less pyoverdin (on a per OD-unit basis), compared to CAA-grown cells. Does this justify the claim that the "collective production"[14] of KB-grown cells is negligible? No it does not. Even if we assume that K&R intended their claim to apply to per capita (or, more accurately, per OD_{600} unit) production, this would be true if and only if the relationship between pyoverdin and cell number was linear. The relationship between these variables for *P. aeruginosa* PAO1 is known, and is anything but linear (see Kümmerli et al. 2009; Kümmerli and Brown 2010).

A nonlinear relationship between growth and pyoverdin stems from complex regulation. Two layers are relevant: the first involves positive feedback between the ferripyoverdin complex and transcription of pyoverdin biosynthetic genes (Lamont et al. 2002; Visca et al. 2007). Such regulation ensures exponential escalation of pyoverdin biosynthesis. The second involves Fur-mediated repression, which means that the total amount of secreted pyoverdin saturates above a certain concentration (Ochsner et al. 1995). Given both layers of regulation, the amount of pyoverdin (in a closed planktonic environment) is expected to plateau at some maximum level. Indeed, provided that available iron levels are low, thus stimulating pyoverdin synthesis—and nutrient limitation not so extreme as to prevent growth—then the threshold level should be independent of the test environment (and the same in every instance). However, cell growth, not being limited by iron (there exists an abundance of pyoverdin) is likely to continue through at least 48 h (in static culture) provided sufficiency of carbon and nitrogen. This is precisely what the data of K&R show. Differences in pyoverdin production per OD unit reported by K&R have nothing to do with differences in the

[10]The QuantiChrom iron assay kit and its contents are proprietary information. Attempts to obtain the identity of constituent components and processes from the manufacturers were declined.

[11]Meyer and Abdallah (1978) confirmed the importance of low iron concentrations for maximization of pigment production: they showed pyoverdin production to be completely repressed in media containing 1 mg L^{-1} Fe(III); levels of Fe(III) below 0.2 mg L^{-1} reduce cell growth and limit pyoverdin production.

[12]K&R perform their experiment using *P. aeruginosa* (and not *P. fluorescens* as used by Z&R) and a culture period of 24 h (and not 48 h as in Z&R).

[13]K&R do not measure viable cells: OD_{600} values ranging from 0.03 to 1.3 correspond to differences of ~2–4 log units.

[14]The use of the term "collective production" is curious, given that total pyoverdin production of cultures (measured as RFU) did not vary between KB and CAA.

Table 1. Effect of medium on growth of *P. fluorescens* and pyoverdin production.

Medium (1)	RFU (2)	CFU (3)
KB	10,385 (9664–11,107)	9.00 (8.98–9.02)
KB plus Fe	−62 (−172–48)	9.46 (9.28–9.64)
KB plus Trf	2041 (1680–2404)	8.44 (8.38–8.49)
CAA	42,051 (39,961–44,141)	8.54 (8.49–8.59)
CAA plus Fe	2089 (1957–2222)	8.31 (8.18–8.43)
CAA plus Trf	453 (361–545)	6.66 (6.65–6.79)

Notes : (1) *Pseudomonas fluorescens* SBW25 cells were grown in 6 mL liquid culture without shaking for 48 h. KB, King's Medium B; KB plus Fe, King's Medium B supplemented with 45 μM $FeSO_4$; KB plus Trf, King's Medium B supplemented with 100 μg mL^{-1} apotransferrin; CAA, casamino acids medium; CAA plus Fe, casamino acids medium supplemented with 45 μM $FeSO_4$; and CAA plus Trf, casamino acids medium supplemented with 100 μg mL^{-1} apotransferrin.

(2) Pyoverdin production expressed as relative fluorescence units and determined by the method of Kümmerli et al. (2009). Data are means and 95% confidence intervals from three biological replicates.

(3) Population density expressed as log10 colony forming units (CFU) mL^{-1} and determined by dilution plating. Data are means and 95% confidence intervals from three biological replicates.

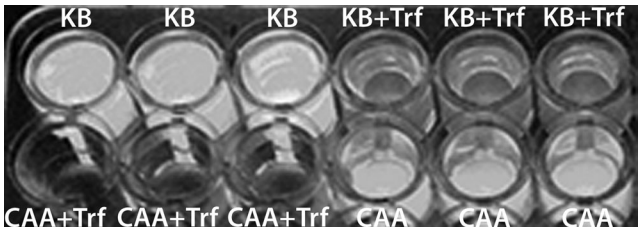

Figure 1. *Pseudomonas fluorescens* SBW25 cells grown in King's Medium B (KB) produce pyoverdin. Portion of a microtiter plate containing culture supernatant from cells grown in KB and CAA viewed under UV light (302 nm), prior to analysis based on specific excitation and emission properties of the molecule (for quantitative data see Table 1). KB, King's Medium B; KB + Trf, King's Medium B supplemented with 100 μg mL^{-1} apotransferrin; CAA, casamino acids medium; CAA + Trf, casamino acids medium supplemented with 100 μg mL^{-1} apotransferrin.

iron status of KB versus CAA. Rather, they are a direct consequence of the vastly different nutritional status of KB compared to CAA.

KB PROMOTES THE PRODUCTION OF PYOVERDIN IN RESPONSE TO LOW LEVELS OF AVAILABLE IRON

We present data from a simple experiment that confirms that production of pyoverdin in KB (by *P. fluorescens* SBW25) is a consequence of low levels of available iron. We grew SBW25 in KB—over a 48-h period in static microcosms—with and without the addition of surplus iron (45 μM $FeSO_4$). Pyoverdin was measured as in K&R, but cell growth was determined by dilution plating. The data are shown in Table 1 (see also Fig. 1). SBW25 grown in KB produces pyoverdin (10,385 RFU [9664–11,107]). No pyoverdin is produced when ferrous iron is added to the medium (−62 RFU [−172–48]). KB therefore promotes production of pyoverdin and production is a direct response to low levels of available iron.

We also cultured SBW25 in CAA with and without ferrous iron. Compared to KB-grown cells, more pyoverdin was detected in microcosms containing CAA grown cells. Pyoverdin was significantly reduced in CAA supplemented with ferrous iron, although did not return to zero. The bacterium was also cultured in KB and CAA containing 100 μg mL^{-1} apotransferrin. A reduction in pyoverdin was noted in both media compared to the same media without apotransferrin. Cell numbers were particularly affected in CAA with apotransferrin. Of note is lack of evidence of a plateau for pyoverdin production by SBW25. This is consistent with Z&R's finding that the ferripyoverdin complex appears able to override Fur-mediated repression in SBW25. This is an intriguing difference compared to PAO1 that warrants additional investigation. Indeed, this and many additional findings reported by Z&R indicate that the relationship between *Pseudomonas*—the production of pyoverdin—and environment is more complex than currently appreciated.

There exists the possibility that fluorescence emitted by pyocyanin may interfere with measurement of pyoverdin. To exclude this possibility, we took note of known fluorescent properties of pyocyanin (Sullivan et al. 2011) and used epifluorescence microscopy to selectively measure pyoverdin (Julou et al. 2013). Microcolonies of *P. fluorescens* SBW25—and for this work we included *P. aeruginosa* PAO1—were grown (separately) for 20 h at 28°C (the optimal temperature for pyoverdin production by PAO1 [Meyer et al. 1996]) on an agar gel sealed with a coverslip. Slides containing microcolonies were transferred to an inverted microscope for quantification of pyoverdin. As shown in Figure 2, KB promotes production of pyoverdin synthesis in both *P. fluorescens* and *P. aeruginosa*, although levels were noticeably higher in *P. fluorescens*. No signal was detected in mutants compromised in their ability to produce pyoverdin, thus supporting the conclusion that the observed fluorescence is solely attributable to pyoverdin (and not pyocyanin). Moreover, both bacteria, when grown on KB, produce pyoverdin.

HOW P. FLUORESCENS OBTAINS IRON IN KB CULTURE

Having confuted the assertions of K&R and shown KB to be as relevant in an environment (albeit a laboratory environment) in which to study pyoverdin as any other, we turn attention to the significance of pyoverdin (in KB), its role in iron metabolism, and

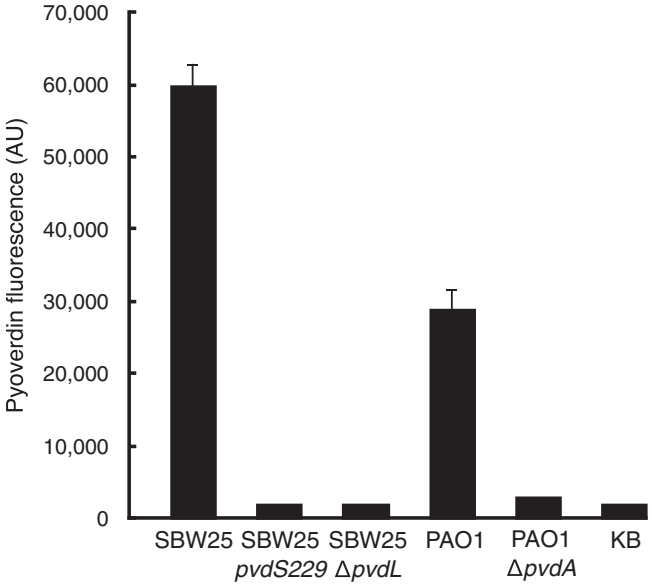

Figure 2. Quantification of pyoverdin production by *P. fluorescens* SBW25 and *P. aeruginosa* PAO1 grown on KB agar slabs. Data are means and standard deviations from seven independent observations per slide. SBW25 *pvdS229*, SBW25 Δ*pvdL*, and PAO1 Δ*pvdA* are nonpyoverdin producing mutants. Control is uninoculated KB.

the causes underpinning the evolutionary success of nonproducing mutants. Z&R give this matter extensive attention.

It is increasingly clear that pyoverdin and its role in iron metabolism is anything but simple—even in KB. The dynamics are not fully captured by simplistic expressions where costs and benefits are fixed, where feedback between organismal activity and environmental change is ignored, and where mechanistic details of regulation are absent. Understanding requires a grasp of chemical, physiological, ecological, and evolutionary factors—and the interplay among them. Moreover, it requires recognition that organisms modify their environment and such modifications feedback to affect physiology and function. It also requires recognition of the nature of the laboratory environment and the possibility that what is observed therein may make little sense from the perspective of organismal performance.

Uninoculated KB is replete in dissolved oxygen and deplete in available iron (iron exists in the Fe(III) state as insoluble polymers or complexed with organic ligands). Upon inoculation into KB, *P. fluorescens* senses the environment to be low in iron and this causes derepression of Fur and activation of pyoverdin biosynthesis. There is no reason to doubt that the bacterium benefits from uptake of the ferripyoverdin complex from which it liberates iron. In spatially structured culture, growth rapidly depletes oxygen from the broth phase, creating a gradient that descends steeply from the air-liquid interface (oxygen is absent from all but the top few micrometers within a few hours growth). There are numerous ensuing effects due to factors such as pH, redox chemistry, and metal solubility. These in turn impact upon bacterial physiology. Whereas pyoverdin likely played an important early role in the acquisition of iron, its importance wanes as growth continues in closed planktonic culture. This is because as oxygen concentrations decrease, iron becomes increasingly soluble and available. At this point a bacterium well tuned to its environment would shut down synthesis of pyoverdin. However, SBW25 is unable to affect this response. This is a consequence of positive feedback between freely diffusible ferripyoverdin and transcriptional activation of pyoverdin synthesis, which in a closed planktonic environment leads to escalating production of pyoverdin. This is maladaptive: it sets the scene for the evolution of nonproducing types.

When pyoverdin-defective (*pvdS*) mutants grow in KB in the absence of producing types, they must necessarily gain iron independently of pyoverdin. This could involve an alternate chelating agent, but it may simply be that there exists the possibility for a modicum of growth sufficient to effect a decrease in oxygen concentration thus promoting reduction of Fe(III) to the soluble Fe(II) form. Under such conditions cellular iron needs are met independently of exogenous chelating agents.

It is important to be clear about the cause of pyoverdin nonproducing types. The cause is a regulatory system that is poorly suited to life in planktonic KB culture. Pyoverdin production itself is not maladaptive (as K&R claim); rather, it is the *regulation* of pyoverdin production. It seems that regulation is not tuned to the demands of growth in structured KB microcosms. Although sobering, with hindsight, this is hardly surprising given that closed KB microcosms are not the natural environment for *P. fluorescens*.

We suspect that the regulation of pyoverdin is generally ill-suited to growth in closed planktonic conditions (Julou et al. 2013)—again, reflecting the fact that the regulation of pyoverdin is likely tuned to conditions encountered by *Pseudomonas* in its natural habitat. There is even reason to suggest that such maladaptive regulation is a factor in the evolution of nonpyoverdin-producing mutants in the cystic fibrosis (see Z&R). It would be of more than passing interest for K&R to repeat the work of Z&R in their focal—CAA supplemented with apotransferrin—medium to see whether nonproducing mutants spontaneously arise, to characterize these mutants, and then to seek an understanding of the causes of their evolution. It would be especially interesting to examine the effect of severe iron limitation (and deleterious impacts on growth) on cell physiology, on regulation of pyoverdin production, and ensuing effects on the nature of the interaction between producing and nonproducing cells. Indeed, we urge K&R to open the black box that surrounds growth, physiology, and genetics of pyoverdin-producing and pyoverdin-nonproducing types (and pyoverdin-mediated interactions among these types) in CAA supplemented with apotransferrin.

The Purpose of Models and Misconceptions of Social Evolution Theory

In the penultimate section of their criticism, K&R assert that Z&R misconceive social evolution theory. The nature of the theory misunderstood is not made clear, but disquieting to K&R is Z&R's finding that pyoverdin can be personalized, although the full significance of this finding seems to have gone unrecognized (see below). Rather than considering the possibility that pyoverdin may, under some conditions, fail to perform as a public good (as the data of Z&R show), K&R sketch a model that shows how the social evolution framework readily accommodates personalized "public goods." We do not dispute that a cooperative trait can be personalized (see, e.g., Spiers et al. 2002; Rainey and Rainey 2003), but the challenge laid down by the data of Z&R is not met by showing that personalization is compatible with the sociobiology framework. The challenge is met when data incompatible with predictions from sociobiology are confronted, alternate explanations considered, and those alternate explanations tested.

Nonetheless the model sketched by K&R is worth examination. First though, it needs to be seen in light of several substantive theoretically informed models of diffusion-mediated sharing of extracellular products (Driscoll and Pepper 2010; Allen et al. 2013; Nadell et al. 2013).[15] These studies show that increased diffusion distance, lower diffusion coefficients, and (in some cases) increased uptake rates serve to increase personalization, thus augmenting the direct fitness of producers. The model of K&R is largely free of physically and biologically meaningful parameters. For example, in contrast to Allen et al. (2013), there is no account of spatial structure, cell shape, diffusion rate, or rates of decay. It is therefore difficult to interpret their sketch in either the context of previous work, or the experiments of Z&R. Indeed, all biological details are subsumed within a single, dimensionless "personalization" parameter, which imposes the constraint that any pyoverdin produced must benefit either the producer or the nonproducer (precluding loss through diffusion or degradation). In essence, the model shows that nonproducers can win against producers even when producers personalize some of the products. This is true. It also shows that when producers personalize too much of the product, nonproducers lose. This is also true. In the latter case, the product is a private good and evolution of production is a simple matter.

[15] Allen et al. provide a theoretical analysis of public good production in which they take into account spatial structure, cell shape, diffusion rate, and rates of decay. They show that conditions for diffusible products to be maintained as cooperative traits are especially stringent and unlikely in mixed culture. This provides additional reason to suspect that what is observed in closed planktonic culture may have little relevance to understanding the ecological significance of pyoverdin in nature.

There is little doubt that the hypothesis that pyoverdin is a public good (in the standard game theoretic sense) fails to incorporate important biological parameters. Nonetheless, the claim has one very considerable merit: it makes straightforward predictions that are testable. Moreover, the claim that pyoverdin is a diffusible public good—accessible to *all* individuals (in a well-mixed environment)—has been made repeatedly. To quote from two reviews on microbes and public goods: a public good is "a resource that is costly to produce, and provides a benefit to all the individuals in the local group or population" (West et al. 2006); "public goods lead to the problem of cooperation because they are metabolically costly to the individual to produce but provide a benefit to all the individuals in the local group or population" (West et al. 2007a). Furthermore, it is not uncommon to find pyoverdin discussed as a public good in context of, and with reference to, Hardin's classic "tragedy of the commons," which centers firmly on the notion of "common resource" (Hardin 1968) (see, e.g., Fig. 1 of West et al. 2007a and Kümmerli and Brown 2010).

The preceding discussion, however, misses the significance of one of several pieces of data that led Z&R to point to evidence of personalization. Z&R examine the ability of producing types to increase in frequency (when rare) in the face of numerically superior populations of nonproducers. The expectation according to sociobiology is that when a diffusible product is costly to produce and the product is equally available to nonproducers as to producers (i.e., it is a public good), then producing types will be incapable of invasion. This expectation holds equally for a personalized cooperative trait—as sketched in the model of K&R—in which that part of the product not retained by the producer is available to nonproducing types.

Z&R show that under certain conditions, for example, well-mixed CAA medium supplemented with chelating agent, that pyoverdin producers have substantially higher fitness than nonproducers—even when rare. Such a finding is incompatible with the hypothesis that (in this medium) pyoverdin is a public good. In terms of the model of K&R, this means that the amount of pyoverdin retained by the producer makes the "cost to benefit" ratio favorable to the producer. As a consequence there is insufficient diffusible pyoverdin for nonproducers to prosper (so they lose). Pyoverdin, under these conditions behaves as neither a public good, nor a cooperative trait, but rather, as a "privatized product," the production of which benefits producing cells sufficiently to yield a relative fitness advantage in coculture with nonproducers.

Interestingly, if instead of CAA supplemented with chelating agent, KB is substituted (supplemented with chelating agent), the opposite result is found: pyoverdin producers fail to invade against a numerically superior population of nonproducers. Thus in shaken KB (but not shaken CAA), pyoverdin

behaves as expected of a public good. Together, these two opposing findings show—as stated by Z&R—that the "social" behavior of pyoverdin is highly sensitive to environment (and also to genotype).

Finally, K&R make a distinction between public goods as envisioned by economists and sociobiologists, noting that economists differentiate based on the scale and symmetry of sharing, whereas sociobiologists use the term "more generally" to apply to secreted products, regardless of position along a public–private continuum. But this tendency to use the term "public goods" interchangeably with "extracellular metabolite" is unfortunate, recent, and entirely preventable; it is also a major conceptual criticism raised in Z&R. As stated above, associating biological traits with the game theoretic public goods game constitutes a testable hypothesis and should not become shorthand for extracellular products in general. In some contexts, an external product may truly act as a public good; in others, it may not. Linking these traits and their social dimensions to ecological context is an important and illuminating focus of ongoing theoretical and empirical research.

Reinterpretation of Data From Z&R

If the claims made by K&R had substance, then reassessment of the findings of Z&R would be appropriate. However, it is wrong to cherry-pick and reinterpret data that supports a given position while ignoring or dismissing findings that run contrary to it. It is difficult to view K&R's reinterpretation of the data of Z&R (see K&R Table 1) as anything other than evidence of confirmation bias (Nickerson 1998). Rather than proceed to put right the numerous misinterpretations, errors in assumption (and omission), and statistical issues arising from selective gathering of data from experiments that were each designed to test different predictions, we leave the interested reader to consult Z&R directly.

Having raised concern of confirmation bias, it seems timely to ask why, when KB is as good a laboratory medium at promoting production of pyoverdin as CAA (and is nutritionally balanced), K&R might insist that CAA with high levels of apotransferin (where growth is severely compromised) is the correct environment for exploring the sociobiology of pyoverdin. Could it be because assays performed in this environment ensure conformity to expectations under social evolutionary theory? Tellingly, K&R state that "... previous studies have focussed on pyoverdin production under strongly iron-limited conditions, where it affords the greatest potential benefits to cooperators and cheats alike." Thus, according to K&R, pyoverdin *is* a cooperative trait and nonproducers *are* cheats. This is apparent when the right medium is used. The right medium is one that ensures the outcome accords with the social evolution framework. Reasoning of this kind is circular and ensures that the hypothesis that pyoverdin is a public good (and cooperative trait) can only ever be affirmed: it can never be rejected. But neither can it be tested.

The Importance of Seeking Alternate Explanations and The Dangers of Anthropomorphism

The hypothesis that the microbial world has evolved such that cooperative behavior is the norm is interesting. But it is a hypothesis. Like any hypothesis it requires careful appraisal. A critical part of the appraisal process is rigorous and unbiased experimentation. Of central importance is the search for alternate explanations—the search for falsifiable as well as confirmatory evidence. In our view, in the context of microbes, the importance of searching for falsifiable evidence has been largely overlooked.

Although there is much yet to be accomplished, the work of Z&R has: (1) provided sound reasons to question the generality of the claim that pyoverdin is a public good; (2) shown that nonproducers—even when they evolve de novo in the presence of producers—need not be social cheats; (3) shown that conformity of producers and nonproducers to the social evolution framework is contingent upon both environment and genotype; (4) drawn attention to the artificial nature of laboratory environments and the need to move future studies to natural settings; (5) highlighted the paucity of rigorous experimental evidence underpinning repeated assertions that the microbial world is inherently cooperative; and (6) shown the ecology, ecophysiology, genetics, and cell biology of pyoverdin to be far richer and more complex than currently recognized (see also Julou et al. 2013).

The route to further progress is not obvious. Even the deceptively simple predictions concerning public goods are in reality experimentally challenging to realize. Much though is likely to be gained from mechanistic studies and a move to natural environments. However, real progress requires objectivity and a relentless search for alternate explanations for both producing and nonproducing types. This, we suggest, will occur most naturally when human-centric language is avoided. Such language plays to an innate tendency to anthropomorphize. Projection of human qualities onto the microbial world restricts the search for alternative explanations. For example, the moment nonproducers are labeled "cheats," then the social evolution framework has been assumed. Cheats must necessarily gain advantage from producing types, which accordingly, must be "cooperators"; the product secreted must necessarily be a "public good." None of these assumptions may be correct. Indeed, it is possible to envisage alternate explanations for producing and nonproducing types, and for secreted

products; however, once the social evolution framework has been applied, this is not easily done. Furthermore, given god-like powers of laboratory scientists, it is a relatively trivial matter to inadvertently contrive laboratory conditions such that producers and nonproducers behave in accord with expectations arising from the social evolution framework. Microbes are not bound by such frameworks, but experimenters can be.

In the search for competing hypotheses to explain nonproducing types, Z&R offered an explanation for nonproducing types in KB, but argued that insights from KB might also have relevance in the case of nonproducing strains in the cystic fibrosis lung. Such an explanation gains momentum given new data on the availability of soluble iron in the cystic fibrosis lung and evidence that *Pseudomonas* gains iron from the soluble ferrous fraction and heme (Hunter et al. 2013; Nguyen et al. 2014).

In a similar spirit, we suggest an alternate explanation for the fact that some (but not all) *Pseudomonas* strains can uptake pyoverdin molecules of structural classes other than the one they produce (Cornelis and Matthijs 2002). This may reflect cooperation and cheating (Lee et al. 2012), but it may be an indirect consequence of phage-mediated predation. Of note is substantive genetic diversity (and evidence of positive selection) at the pyoverdin locus; curiously most is confined to a single gene, *fpvA*, which encodes the major ferripyoverdin receptor (Smith et al. 2005). Pyocins (a kind of bacteriocin) gain entry to *Pseudomonas* via the FpvA receptor (Bayasse et al. 1999), as do bacteriophage (Smith et al. 2005). Both chemical warfare among microbes (Czaran et al. 2002; Kerr et al. 2002) and antagonistic coevolution between phage and their hosts (Buckling and Rainey 2002) drive diversification. The capacity of some strains to use unrelated pyoverdin molecules synthesized by competing microbes may simply be a consequence of diversification driven by warfare, combined with functional constraints on the need for uptake of pyoverdin. This hypothesis suggests new opportunities for research.

Conclusion

An important and overarching goal for microbiology is to place knowledge of organismal function in an ecological and evolutionary context within which the biology of microbes makes sense. The appropriateness of this context is critical because it impacts on how the microbial world is conceived—the kinds of questions asked and the types of experiments performed (Redfield 2002). It is especially important in the face of the current drive to understand microbial communities, treat disease, develop new tools for environmental application and in biotechnology.

Microbial communities are shaped by interactions among constituent members. Interactions within and among genotypes come in a great many guises. While some interactions fall within the scope of sociobiology, many others do not. Indeed, one unanticipated outcome of the work of Z&R is recognition of the extreme sensitivity of interactions to subtle changes in environment and genotype. An interaction that might appear to conform to a social dilemma under one set of conditions can change entirely upon a small shift in the nutritional status of the environment. Interactions are rich and complex and a door to numerous research opportunities.

Progress requires studies that shed light on the nature of interactions, their scale, stability, genotype/environment dependency, and fluidity. Such studies are likely to be most productive when performed in an objective manner and free from expectations driven by a theoretical framework whose general relevance to microbes remains to be demonstrated. As mechanistic understanding increases, it will become possible to describe interactions in terms of biological detail and fitness effects (and consequences) without need to resort to the constraints of anthropomorphic language. From such advances a substantive theory of interactions stands to emerge.

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DATA ARCHIVING

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LITERATURE CITED

Allen, B., J. Gore, and M. A. Nowak. 2013. Spatial dilemmas of diffusible public goods. Elife 2:e01169.

Bayasse, C., J. M. Meyer, P. Plesiat, V. Geoffroy, Y. Michel-Briand, and P. Cornelis. 1999. Uptake of pyocin S3 occurs through the outer membrane ferripyoverdine type II receptor of *Pseudomonas aeruginosa*. J. Bacteriol. 181:3849–3851.

Braud, A., V. Geoffroy, F. Hoegy, G. L. A. Mislin, and I. Schalk. 2010. The siderophores pyverdine and pyochelin are involved in *Pseudomonas aeruginosa* resistance against metals: another biological function of these two siderophores. Environ. Microbiol. Rep. 2:419–425.

Buckling, A., and P. B. Rainey. 2002. Antagonistic coevolution between a bacterium and a bacteriophage. Proc. R. Soc. Lond. B 269:931–936.

Chiadò, A., L. Varani, F. Bosco, and L. Marmo. 2013. Opening study on the development of a new biosensor for metal toxicity based on *Pseudomonas fluorescens* pyoverdine. Biosensors 3:385–399.

Cornelis, P., and S. Matthijs. 2002. Diversity of siderophore-mediated iron uptake systems in fluorescent pseudomonads: not only pyoverdines. Environ. Microbiol. 4:787–798.

Czaran, T. L., R. F. Hoekstra, and L. Pagie. 2002. Chemical warfare between microbes promotes biodiversity. Proc. Natl. Acad. Sci. USA 99:786–790.

Dionisio, F., and I. Gordo. 2006. The tragedy of the commons, the public goods dilemma, and the meaning of rivalry and excludability in evolutionary biology. Evol. Ecol. Res. 8:321–332.

Driscoll, W. W., and J. W. Pepper. 2010. Theory for the evolution of diffusible external goods. Evolution 64:2682–2687.

Driscoll, W. W., N. J. Espinosa, O. T. Eldakar, and J. D. Hackett. 2013. Allelopathy as an emergent, exploitable public good in the bloom-forming microalga *Prymnesium parvum*. Evolution 67:1582–1590.

Foster, K. R. 2010. Social behaviour in microorganisms. Pp. 331–356 *in* T. Székely, A. J. Moore, and J. Komdeur, eds. Social behaviour: genes, ecology and evolution. Cambridge Univ. Press, Cambridge, U.K.

Garibaldi, J. A. 1967. Media for the enhancement of fluorescent pigment production by *Pseudomonas* species. J. Bacteriol. 94:1296–1299.

Georgia, F. R., and C. F. Poe. 1932. Study of bacterial fluorescence in various media. II. The production of fluorescence in media made from peptones. J. Bacteriol. 23:135–145.

Gessard, C. 1892. Sur la function fluorescigène des microbes. Ann. de i'Inst. Pasteur 6:801–823.

Griffin, A. S., S. A. West, and A. Buckling. 2004. Cooperation and competition in pathogenic bacteria. Nature 430:1024–1027.

Hallberg, L. 1981. Bioavailability of dietary iron in man. Annu. Rev. Nutr. 1:123–147.

Hannauer, M., A. Braud, F. Hoegy, P. Ronot, A. Boos, and I. J. Schalk. 2011. The PvdRT-OpmQ efflux pump controls the metal selectivity of the iron uptake pathway mediated by the siderophore pyoverdine in *Pseudomonas aeruginosa*. Environ. Microbiol. 14:1696–1708.

Hardin, G. 1968. The tragedy of the commons. Science 162:1243–1248.

Hassett, D. J., P. A. Sokol, M. L. Howell, J. F. Ma, H. T. Schweizer, U. Ochsner, and M. L. Vasil. 1996. Ferric uptake regulator (Fur) mutants of *Pseudomonas aeruginosa* demonstrate defective siderophore-mediated iron uptake, altered aerobic growth, and decreased superoxide dismutase and catalase activities. J. Bacteriol. 178:3996–4003.

Hohnadel, D., and J. M. Meyer. 1988. Specificity of pyoverdine-mediated iron uptake among fluorescent Pseudomonas strains. J. Bacteriol. 170:4865–4873.

Hunter, R. C., F. Asfour, J. Dingemans, B. L. Osuna, T. Samad, A. Malfroot, P. Cornelis, and D. K. Newman. 2013. Ferrous iron is a significant component of bioavailable iron in cystic fibrosis airways. mBio 4:e00557–00513.

Joyner, D. C., and S. E. Lindow. 2000. Heterogeneity of iron bioavailability on plants assessed with a whole-cell GFP-based bacterial biosensor. Microbiology 146:2435–2445.

Julou, T., T. Mora, L. Guillon, V. Croquette, I. J. Schalk, D. Bensimon, and N. Desprat. 2013. Cell-cell contacts confine public goods diffusion inside *Pseudomonas aeruginosa* clonal microcolonies. Proc. Natl. Acad. Sci. USA 110:12577–12582.

Kerr, B., M. A. Riley, M. W. Feldman, and B. J. M. Bohannan. 2002. Local dispersal promotes biodiversity in a real-life game of rock-paper-scissors. Nature 418:171–174.

King, E. O., M. K. Ward, and D. C. Raney. 1954. Two simple media for the demonstration of pyocyanin and fluorescin. J. Lab. Clin. Med. 44:301–307.

Kraemer, S. M. 2004. Iron oxide dissolution and solubility in the presence of siderophores. Aquat. Sci. 66:3–18.

Kuhn, T. S. 1962. The structure of scientific revolutions. The University of Chicago Press, Chicago.

Kümmerli, R., and S. P. Brown. 2010. Molecular and regulatory properties of a public good shape the evolution of cooperation. Proc. Natl. Acad. Sci. USA 107:18921–18926.

Kümmerli, R., and A. Ross-Gillespie. 2013. Explaining the sociobiology of pyoverdin producing *Pseudomonas*: a comment on Zhang and Rainey (2013). Evolution 68:3337–3343.

Kümmerli, R., N. Jiricny, L. Clarke, S. West, and A. Griffin. 2009. Phenotypic plasticity of a cooperative behaviour in bacteria. J. Evol. Biol. 22:589–598.

Lamont, I. L., P. A. Beare, U. Ochsner, A. I. Vasil, and M. L. Vasil. 2002. Siderophore-mediated signaling regulates virulence factor production in *Pseudomonas aeruginosa*. Proc. Natl. Acad. Sci. USA 99:7072–7077.

Lee, W., M. van Baalen, and V. A. A. Jansen. 2012. An evolutionary mechanism for diversity in siderophore-producing bacteria. Ecol. Lett. 15:119–125.

Lippard, S. J., and J. M. Berg. 1994. Principles of bioinorganic chemistry. University Science Books, Sausalito, CA.

Loper, J. E., and S. E. Lindow. 1994. A biological sensor for iron available to bacteria in their habitats on plant-surfaces. Appl. Environ. Microbiol. 60:1934–1941.

Meyer, J.-M., A. Neely, A. Stintzi, C. Georges, and I. A. Holder. 1996. Pyoverdin is essential for virulence of *Pseudomonas aeruginosa*. Infect. Immun. 64:518–523.

Meyer, J. M., and M. A. Abdallah. 1978. The fluorescent pigment of *Pseudomonas fluorescens*: biosynthesis, purification and physicochemical properties. J. Gen. Microbiol. 107:319–328.

Nadell, C. D., J. B. Xavier, and K. R. Foster. 2009. The sociobiology of biofilms. FEMS Microbiol. Rev. 33:206–224.

Nadell, C. D., V. Bucci, K. Drescher, S. A. Levin, B. L. Bassler, and J. B. Xavier. 2013. Cutting through the complexity of cell collectives. Proc. R. Soc. Lond. B 280:20122770.

Neilands, J. B. 1981. Microbial iron compounds. Annu. Rev. Biochem. 50:715–731.

Nguyen, A. T., M. J. O'Neill, A. M. Watts, C. L. Robson, I. L. Lamont, A. Wilks, and A. G. Oglesby-Sherrouse. 2014. Adaptation of iron homeostasis pathways by a *Pseudomonas aeruginosa* pyoverdine mutant in the cystic fibrosis lung. J. Bacteriol. 196:2265–2276.

Nickerson, R. S. 1998. Confirmation bias: a ubiquitous phenomenon in many guises. Rev. Gen. Psychol. 2:175–220.

Ochsner, U., A. Vasil, and M. Vasil. 1995. Role of the ferric uptake regulator of *Pseudomonas aeruginosa* in the regulation of siderophores and exotoxin a expression: purification and activity on iron-regulated promoters. J. Bacteriol. 177:7194–7201.

Olson, M. 1965. The logic of collective action: public goods and the theory of groups. Harvard Univ. Press, Cambridge, MA.

Paton, A. M. 1959. Enhancement of pigment production by *Pseudomonas*. Nature 184:1254.

Popper, K. R. 1959. The logic of scientific discovery. Hutchinson, London.

Rainey, P. B., and K. Rainey. 2003. Evolution of cooperation and conflict in experimental bacterial populations. Nature 425:72–74.

Redfield, R. J. 2002. Is quorum sensing a side effect of diffusion sensing? Trends Microbiol. 10:365–370.

Rue, E. L., and K. W. Bruland. 1995. Complexation of iron(III) by natural organic-ligands in the central North Pacific as determined by a new competitive ligand equilibration adsorptive cathodic stripping voltammetric method. Mar. Chem. 50:117–138.

Schalk, I. J., M. Hannauer, and A. Braud. 2011. New roles for bacterial siderophores in metal transport and tolerance. Environ. Microbiol. 13:2844–2854.

Smith, E. E., E. H. Sims, D. H. Spencer, R. Kaul, and M. V. Olson. 2005. Evidence for diversifying selection at the pyoverdine locus of *Pseudomonas aeruginosa*. J. Bacteriol. 187:2138–2147.

Spiers, A. J., S. G. Kahn, J. Bohannon, M. Travisano, and P. B. Rainey. 2002. Adaptive divergence in experimental populations of *Pseudomonas fluorescens*. I. Genetic and phenotypic bases of wrinkly spreader fitness. Genetics 161:33–46.

Stanier, R. Y., N. J. Palleroni, and M. Doudoroff. 1966. The aerobic pseudomonads: a taxonomic study. J. Gen. Microbiol. 43:159–271.

Sullivan, N. L., D. S. Tzeranis, Y. Wang, P. T. So, and D. Newman. 2011. Quantifying the dynamics of bacterial secondary metabolites by spectral multiphoton microscopy. ACS Chem. Biol. 6:893–899.

Visca, P., F. Imperi, and I. L. Lamont. 2007. Pyoverdine siderophores: from biogenesis to biosignificance. Trends Microbiol. 15:22–30.

West, S. A., and A. Buckling. 2003. Cooperation, virulence and siderophore production in bacterial parasites. Proc. R. Soc. B 270:37–44.

West, S. A., A. S. Griffin, A. Gardner, and S. P. Diggle. 2006. Social evolution theory for microorganisms. Nat. Rev. Microbiol. 4:597–607.

West, S. A., S. P. Diggle, A. Buckling, A. Gardner, and A. S. Griffin. 2007a. The social lives of microbes. Annu. Rev. Ecol. Evol. Syst. 38:53–77.

West, S. A., A. S. Griffin, and A. Gardner. 2007b. Social semantics: altruism, cooperation, mutualism, strong reciprocity and group selection. J. Evol. Biol. 20:415–432.

Zhang, X.-X., and P. B. Rainey. 2013. Exploring the sociobiology of pyoverdin-producing *Pseudomonas*. Evolution 67:3161–3174.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Table S1. Cell growth and pyoverdin production.