Supplemental Appendix For:

Social cheating in a *Pseudomonas aeruginosa* quorum-sensing variant

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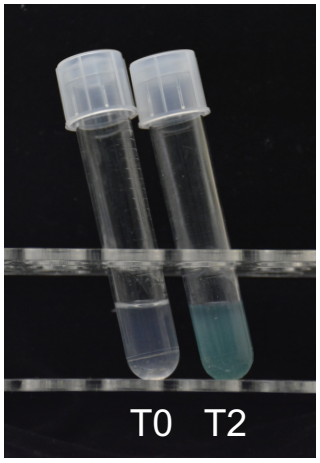

Figure S1. Growth of strain E80 in casein broth. Shown are images of casein broth cultures just after inoculation with E80 overnight culture in LB-MOPS (T0) and after approximately 2 days of growth (T2). At inoculation time the tube is clear, after 2-days growth the bacteria have proteolyzed and consumed the caseinate and the tube is turbid and blue green from production of pyocyanin during growth. When inoculation is conducted from well-developed casein broth culture to fresh casein broth, growth time is shortened to about 24 h.

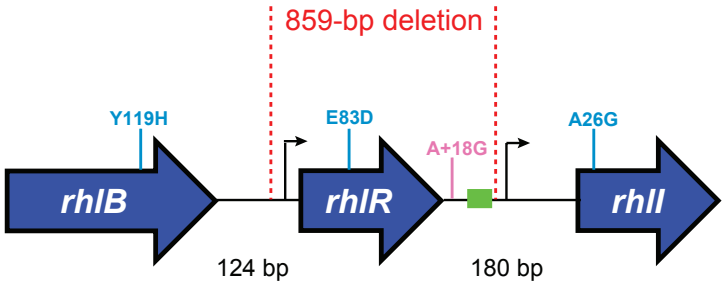

Figure S2. The *rhlRI* region of the chromosome in the E80 parent and RhlRI$^-$ mutant. A comparison of DNA sequences between E80 and PAO1 in the *rhlRI* region revealed several differences. Those sequence differences resulting in an amino acid change in the polypeptide are indicated in teal-colored text above the open reading frame (blue arrow); the single difference in non-coding DNA occurs 18 bases beyond the *rhlR* stop codon (pink-colored text). The 859-bp region lacking in the RhlRI$^-$ mutant is indicated by the red dashed lines. The deletion includes part of the *rhlI* promoter and all of the LasR/RhlR-binding site (green box) required for full transcription of the *rhlI* gene (1). The transcriptional start sites are indicated by black arrows (1, 2).

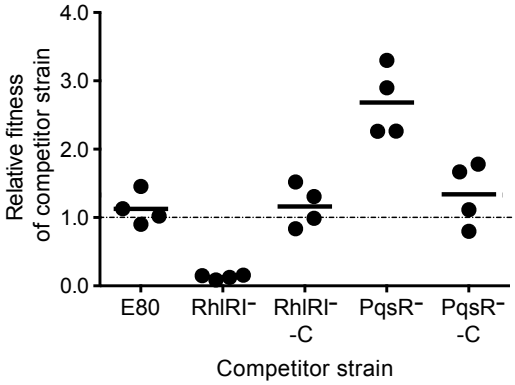

Figure S3. Competition of the PqsR mutant, the RhlRI mutant and the parent strain E80 in casein broth. Performing these experiments required some modifications as RhlRI and PqsR mutants cannot grow individually in casein broth and the E80 parent does not initially grow well in casein broth when inoculated from LB-grown cultures. In order to "jump start" these competitions, we included 100 μl of cell-free supernatant fluid from a densely grown (48 h) E80 casein culture and all inocula were prepared from log-phase LB-MOPS cultures (OD_{600} 0.8). The starting percentage of the competitors was 10%. Each symbol represents the competitive index of competitor strain against the parent strain (E80 or E80-Gm) after 24 h in casein broth; the solid lines represent means for each group. The outcomes above the dashed line indicate the competitor had a fitness advantage and below indicate the parent E80 had a fitness advantage. As in the LB-MOPS competitions (Fig. 5B), the RhlRI mutant was less fit than the parent and the $PqsR^-$ mutant was more fit than the parent.

Table S1. Strains used in this study

Strain	Relevant characteristic(s) [a]	Source
P. aeruginosa		
PAO1	Wildtype	(3)
PAO1 LasR$^-$	PAO1 with an unmarked in-frame *lasR* deletion	(4)
PAO1 RhlR$^-$	PAO1 with an unmarked in-frame *rhlR* deletion	(4)
E80	Isolated from CF patient sputum, Δ321-324 deletion in *lasR*	(5)
E80-Gm	E80 with a gentamicin-resistance cassette at *att* site, Gen^R	This work
E80 RhlRI$^-$	E80 with an unmarked *rhlR* and P_{rhlI} deletion (see Fig. S2)	This work
E80 RhlRI$^-$-C	E80 RhlRI$^-$ complemented with wildtype *rhlRI* at *att* site, Gen^R	This work
E80 PqsR$^-$	E80 with an unmarked *pqsR* deletion	This work
E80 PqsR$^-$-C	PqsR$^-$ complemented with wildtype *pqsR* at *att* site, Gen^R	This work
1	Evolved E80 freeloader, Δ371-374 deletion in *pqsR*	This work
2	Evolved E80 freeloader, Δ109-121 deletion in *pqsR*	This work
3	Evolved E80 freeloader, T811G substitution in *pqsR*	This work
4	Evolved E80 freeloader, C101T substitution in *pqsR*	This work
5	Evolved E80 freeloader, wildtype *pqsR*	This work
6	Evolved E80 freeloader, wildtype *pqsR*	This work
1-C	Freeloader (E80) 1 complemented with wildtype *pqsR* at *att* site	This work
2-C	Freeloader (E80) 2 complemented with wildtype *pqsR* at *att* site	This work
E. coli		
DH5α	*fhuA2 Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	Invitrogen
S17	*thi, pro, hdsR, hdsM+ recA,* RP4-2 (Tc::Mu Km::Tn7)	(6)
MG4 pECP61.5	C4-HSL bioassay reporter strain; Amp^R	(7)
HB101 pRK2013	Conjugal mating helper strain, Km^R	(8)

[a]Resistant to gentamicin (Gen^R), ampicillin (Amp^R) or kanamycin (Kan^R)

Table S2. Plasmids and primers used in this study

Plasmid or primer	Relevant characteristic(s) [a]	Source
Plasmids		
pTNS2	Source of transposase, Amp^R	(9)
pFLP2	Source of Flp recombinase, Amp^R/Cb^R	(9)
pUC18-mini-Tn7T-Gm	mini-Tn7 chromosomal gene integration vector, Amp^R/Gen^R	(9)
pEXG2	Suicide vector, Gen^R	(10)
pEXG2-rhlRI	Plasmid for RhlRI-mutant strain construction (see Fig. S2), Gen^R	This work
pEXG2-pqsR	Plasmid for PqsR-mutant strain construction (-90 to +730 bp), Gen^R	This work
Tn7-pqsR	mini-Tn7 containing DNA from 255 upstream of the *pqsR* start codon to 136 bp downstream of the *pqsR* stop codon for complementation, Amp^R/Gen^R	This work
Tn7-rhlRI	mini-Tn7 containing DNA from 129 bp upstream of the *rhlR* start codon to 154 bp downstream of the *rhlI* stop codon for complementation, Amp^R/Gen^R	This work
Primers		
rhlRI-KO-UP-F	5'-ACGAACTGCAACGCTTTCT-3'	This work
rhlRI-KO-UP-R	5'-TCATGGCAACCCTATCTGTTAT-3'	This work
rhlRI-KO-DN-F	5'-CTCATGTGTGTGCTGGTATGT-3'	This work
rhlRI-KO-DN-R	5'-GTCGAACTGGTCGAATTCCT-3'	This work
pqsR-KO-UP-F	5'-GCCTGTACCAGGAGTCGTTC-3'	This work
pqdR-KO-UP-R	5'-ACTGTGGAAGAAGCGTCGAG-3'	This work
pqsR-KO-DN-F	5'-TCAGCGAACTCTACGAACCG-3'	This work
pqsR-KO-DN-R	5'-GGCAACCTCGATACCAGCAT-3'	This work
rhlRI-Compl-F	5'-CCTGAACGGTGCTGGCATAACA-3'	This work
rhlRI-Compl-R	5'-GGACGAGATGGCGGAATGAC-3'	This work
pqsR-Compl-F	5'-TCACCTCCAAAACGACGACT-3'	This work
pqsR-Compl-R	5'-CGGAAGGTTTCGACTGCCTG-3'	This work

[a] Resistant to ampicillin (Amp^R), carbenicillin (Cb^R), or gentamicin (Gen^R)

SUPPLEMENTAL REFERENCES

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