Supplemental Information for:

Allopatric divergence limits cheating range and alters genetic requirements for a cooperative trait

Kaitlin A. Schaal*, Yuen-Tsu Nicco Yu, Marie Vasse, and Gregory J. Velicer

Institute of Integrative Biology, ETH Zürich

* Corresponding author. Email: kaitlin.schaal@env.ethz.ch

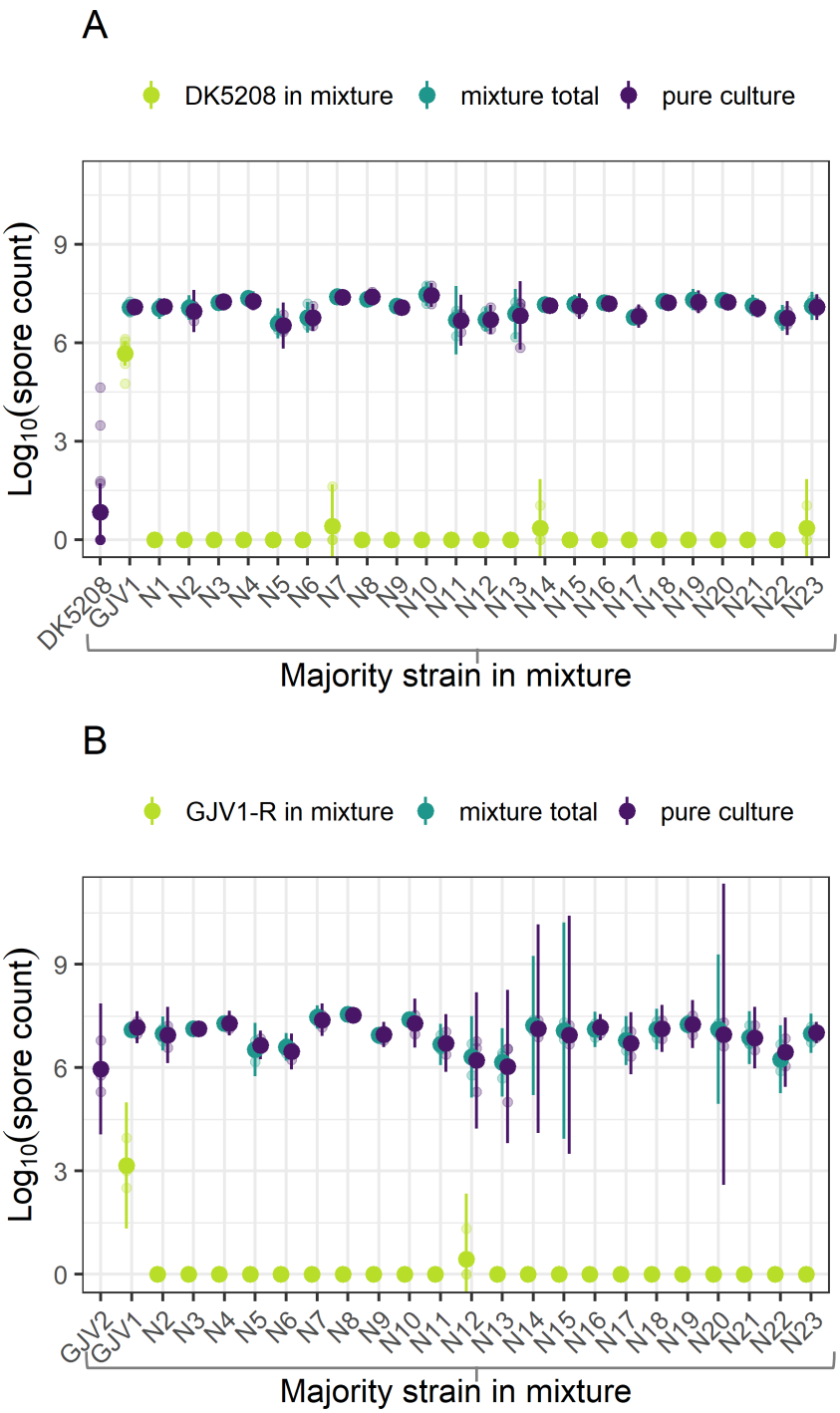

Fig. S1. Absolute sporulation levels of DK5208, GJV1 and 23 natural isolates in pure culture, DK5208 in mixture with all other strains and total spore production of all mixes. (A) DK5208 in mixture with GJV1 and 23 natural isolates. (B) Rifampicin-marked version of GJV1 in mixture with GJV1 and 22 natural isolates. DK5208 and GJV2 started as 1% in all mixed groups. Small circles represent individual replicate estimates, large circles show cross-replicate averages, and error bars show 95% confidence intervals; 3 or 4 biological replicates for natural isolate mixes, 8 for GJV1.

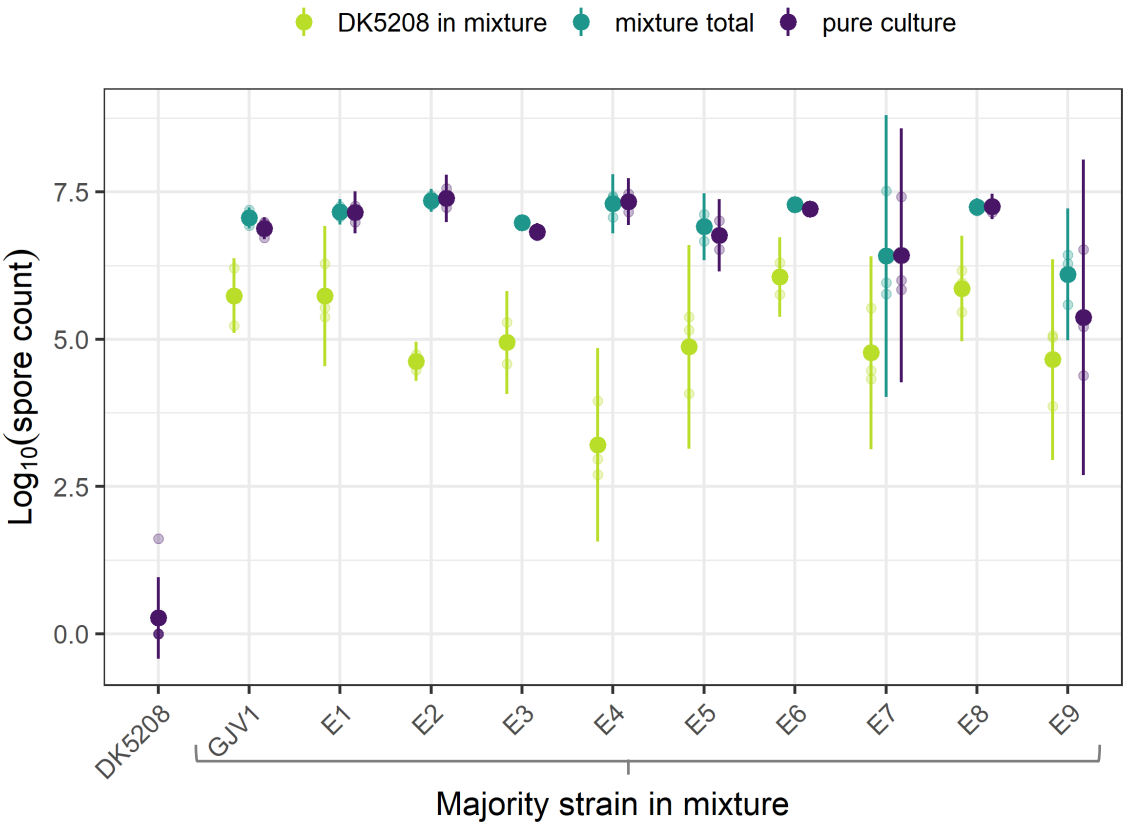

Fig. S2. Absolute sporulation levels of DK5208, GJV1, and all MyxoEE-3 lab-evolved clones in pure culture, DK5208 in mixture with all other strains, and total spore production of all mixes. DK5208 started as 1% in all mixed groups. Small circles represent individual replicate estimates, large circles show cross-replicate averages, and error bars show 95% confidence intervals; 3-4 biological replicates.

A

B

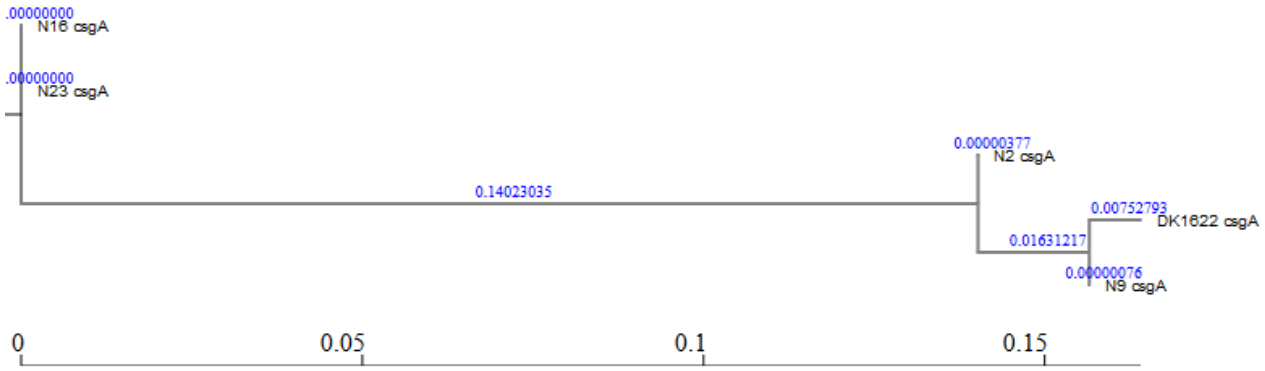

Fig. S3. *csgA* sequences of *M. xanthus* strains DK1622/GJV1 and four natural isolates disrupted at *csgA* in this study. (A) Sequence alignment, where red shading indicates polymorphic sites, with darkness reflecting the degree of polymorphism. Boxed regions indicate the beginning and end of the region used as inserts in plasmid construction. (B) Maximum likelihood tree of *csgA* sequences, where blue numbers indicate branch lengths in substitutions per site. The scale bar is in substitutions per site.

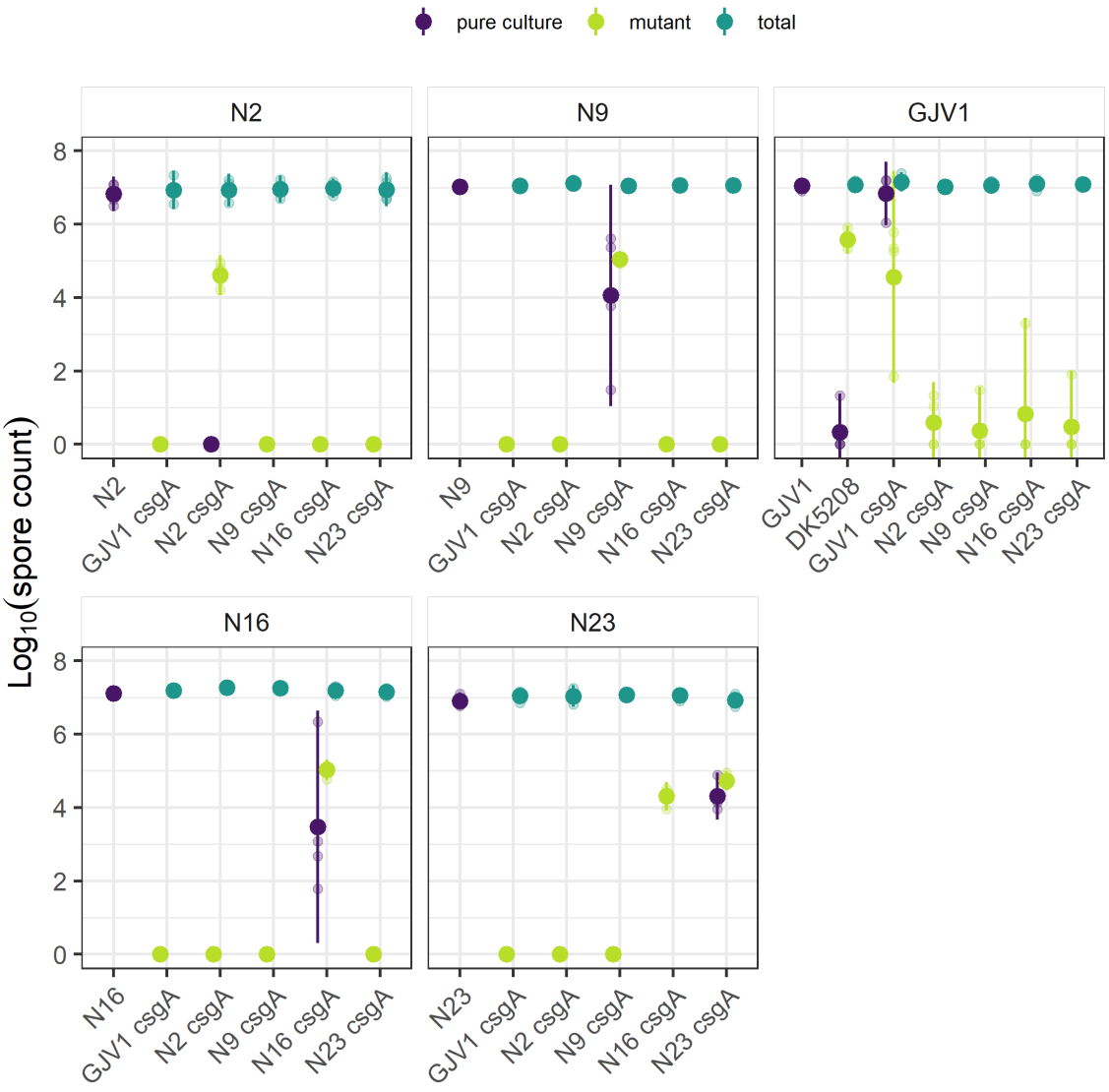

Fig. S4. Absolute sporulation levels of i) DK5208, GJV1, and four natural isolates in pure culture, ii) each of five new c*sgA* mutants in 1:99 mixture with all five parental strains, and 3) total spore production of all mixes. Small circles represent individual replicate estimates, large circles show cross-replicate averages, and error bars show 95% confidence intervals; 4 biological replicates.

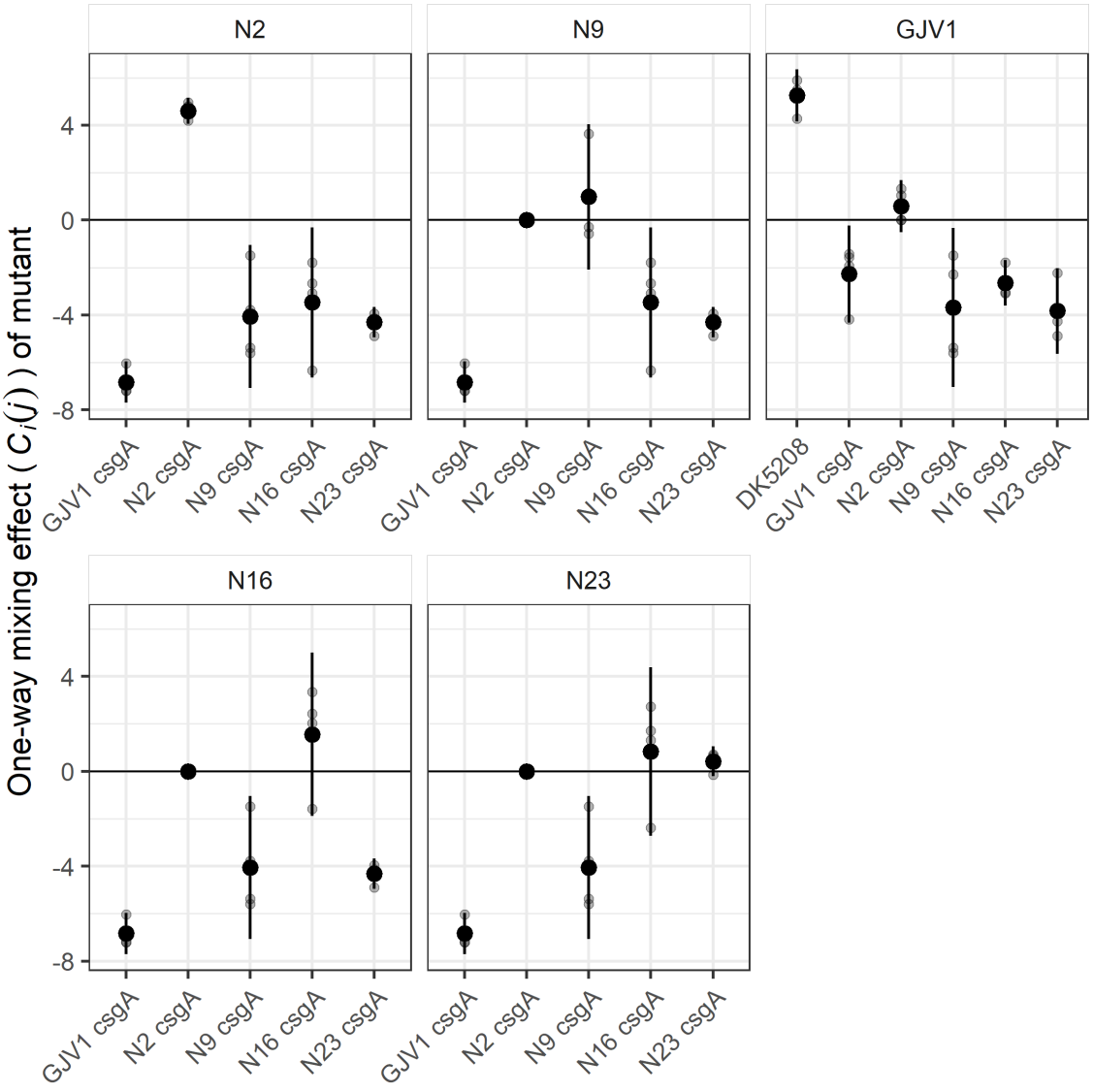

Fig. S5. One-way mixing effects on *csgA* mutants. We calculated the degree to which each *csgA* mutant sporulated better or worse in mixture with each proficient strain than it did in pure culture with the parameter $C_i(j)$ (see Methods). Error bars are 95% confidence intervals. Black dots are averages of four biological replicates (grey dots).

Table S1. *M. xanthus* strains. Here we list the original published or new nomenclature of each strain and the simplified nomenclature used in this paper.

Original or formal name	Ref.	Referred to here as	Genetic manipulation	Resistance	Geographic origin
DK5208/LS523	(1, 2)	DK5208	*csgA*::Tn5-132	oxytetracycline	[a]
GJV1	(3, 4)	GJV1	-	-	[a]
GJV2	(3, 5, 6)	GJV2	spontaneous rif^R mutation	rifampicin	-
MyxoEE-3 P02 cycle 40 clone 1 [b]	(7–9)	E1	-	rifampicin	-
MyxoEE-3 P03 cycle 40 clone 1	(7–9)	E2	-	-	-
MyxoEE-3 P04 cycle 40 clone 1	(7–9)	E3	-	rifampicin	-
MyxoEE-3 P10 cycle 40 clone 1	(7–9)	E4	-	rifampicin	-
MyxoEE-3 P12 cycle 40 clone 1	(7–9)	E5	-	rifampicin	-
MyxoEE-3 P32 cycle 40 clone 1	(7–9)	E6	-	rifampicin	-
MyxoEE-3 P36 cycle 40 clone 1	(7–9)	E7	-	rifampicin	-
MyxoEE-3 P38 cycle 40 clone 2	(7–9)	E8	-	rifampicin	-
MyxoEE-3 P40 cycle 40 clone 1	(7–9)	E9	-	rifampicin	-
Chihaya 01	(10)	N1	-	-	Japan
Chihaya 20	(10)	N2	-	-	Japan
Colombia 01	(10)	N3	-	-	Colombia
Colombia 03	(10)	N4	-	-	Colombia
Nei 05	(10)	N5	-	-	Mongolia
Nei 10	(10)	N6	-	-	Mongolia
New Jersey 06	(10)	N7	-	-	New Jersey, USA
New Jersey 10	(10)	N8	-	-	New Jersey, USA
Serengeti 01	(10)	N9	-	-	Tanzania
Serengeti 21	(10)	N10	-	-	Tanzania
Sulawesi 08	(10)	N11	-	-	Indonesia
Tubingen C22	(10)	N12	-	-	Germany
Tubingen C42	(10)	N13	-	-	Germany
GH2.1.4c40	(11)	N14	-	-	Indiana, USA (“Greg’s House”)
GH3.2.7C	(11)	N15	-	-	Indiana, USA (“Greg’s House”)
GH3.5.6c2	(11)	N16	-	-	Indiana, USA (“Greg’s House”)
GH5.1.9c20	(11)	N17	-	-	Indiana, USA (“Greg’s House”)
KF2.1.1B	(11)	N18	-	-	Indiana, USA (“Kent Farms”)

KF3.2.8c11	(11)	N19	-	-	Indiana, USA (“Kent Farms”)
KF5.4.6c29	(11)	N20	-	-	Indiana, USA (“Kent Farms”)
MC3.1.9c3	(11)	N21	-	-	Indiana, USA (“Moore’s Creek”)
MC3.2.6B	(11)	N22	-	-	Indiana, USA (“Moore’s Creek”)
MC3.5.9c15	(11)	N23	-	-	Indiana, USA (“Moore’s Creek”)
GJV1_*csgA483*	this work	-	*csgA*::pCR-*csgA483*	kanamycin	-
Chihaya 20_*csgA483*	this work	N2 *csgA*	*csgA*::pCR-*csgA483*	kanamycin	-
Serengeti 01_*csgA483*	this work	N9 *csgA*	*csgA*::pCR-*csgA483*	kanamycin	-
GH3.5.6c2_*csgA483*	this work	N16 *csgA*	*csgA*::pCR-*csgA483*	kanamycin	-
MC3.5.9c15_*csgA483*	this work	N23 *csgA*	*csgA*::pCR-*csgA483*	kanamycin	-

[a]Strain DK1622 – the lab progenitor of both DK5208 and GJV1 – is reported to be derived from a strain isolated in Ames, Iowa, USA (12).
[b]Multiple clones were selected and stored frozen after MyxoEE-3 cycle 40.

Table S2. History and mutations of MyxoEE-3 lab-evolved strains. The strains referred to here as E1-E9 are clones from populations of *M. xanthus* that evolved as vegetatively growing colonies swarming across hard (1.5%) or soft (0.5%) agar (7, 8) over 40 two-week growth cycles. Here we provide information about the evolution conditions and accumulated mutations of each strain (from Table S4 in (7)).

Strain	Original name	Agar concentration	Mutations	Genic	Coding	Synonymous	Intergenic
E1	P02 clone 1	1.5%	13	10	8	2	3
E2	P03 clone 1	1.5%	10	10	10	0	0
E3	P04 clone 1	1.5%	10	9	9	0	1
E4	P10 clone 1	1.5%	10	10	7	3	0
E5	P12 clone 1	1.5%	19	15	14	1	4
E6	P32 clone 1	0.5%	12	12	11	1	0
E7	P36 clone 1	0.5%	14	14	13	1	0
E8	P38 clone 2	0.5%	10	10	9	1	0
E9	P40 clone 1	0.5%	13	12	11	1	1

Table S3. Plasmids constructed in this work.

Plasmid name	*csgA* allele
pGJV1*csgA483*	GJV1
pChihaya20*csgA483*	Chihaya 20
pSerengeti01*csgA483*	Serengeti 01
pGH356c2*csgA483*	GH3.5.6c2
pMC359c15*csgA483*	MC3.5.9c15

SI References

1. L. J. Shimkets, S. J. Asher, Use of recombination techniques to examine the structure of the csg locus of Myxococcus xanthus. *Mol. Gen. Genet.* **211**, 63–71 (1988).
2. P. Manhes, G. J. Velicer, Experimental evolution of selfish policing in social bacteria. *Proc. Natl. Acad. Sci.* **108**, 8357–62 (2011).
3. G. J. Velicer, L. Kroos, R. E. Lenski, Loss of social behaviors by Myxococcus xanthus during evolution in an unstructured habitat. *Proc. Natl. Acad. Sci.* **95**, 12376–80 (1998).
4. G. J. Velicer, *et al.*, Comprehensive mutation identification in an evolved bacterial cooperator and its cheating ancestor. *Proc. Natl. Acad. Sci.* **103**, 8107–8112 (2006).
5. F. Fiegna, Y. N. Yu, S. V Kadam, G. J. Velicer, Evolution of an obligate social cheater to a superior cooperator. *Nature* **441**, 310–314 (2006).
6. P. C. Zee, *et al.*, A shift from magnitude to sign epistasis during adaptive evolution of a bacterial social trait. *Evolution (N. Y).* **68**, 2701–2708 (2014).
7. O. Rendueles, *et al.*, Rapid and widespread de novo evolution of kin discrimination. *Proc. Natl. Acad. Sci.* **112**, 9076–81 (2015).
8. O. Rendueles, G. J. Velicer, Evolution by flight and fight: Diverse mechanisms of adaptation by actively motile microbes. *ISME J.* **11**, 555–568 (2017).
9. O. Rendueles, G. J. Velicer, Hidden paths to endless forms most wonderful: Complexity of bacterial motility shapes diversification of latent phenotypes. *BMC Evol. Biol.* **20**, 145 (2020).
10. R. Rajagopalan, S. Wielgoss, G. Lippert, G. J. Velicer, L. Kroos, devI is an Evolutionarily Young Negative Regulator of Myxococcus xanthus Development. *J. Bacteriol.* **197**, JB.02542-14 (2015).
11. S. A. Kraemer, G. J. Velicer, Endemic social diversity within natural kin groups of a cooperative bacterium. *Proc. Natl. Acad. Sci.* **108**, 10823–30 (2011).
12. A. Dey, *et al.*, Sibling rivalry in Myxococcus xanthus is mediated by kin recognition and a polyploid prophage. *J. Bacteriol.* **198**, 994–1004 (2016).