Supplemental Material for:

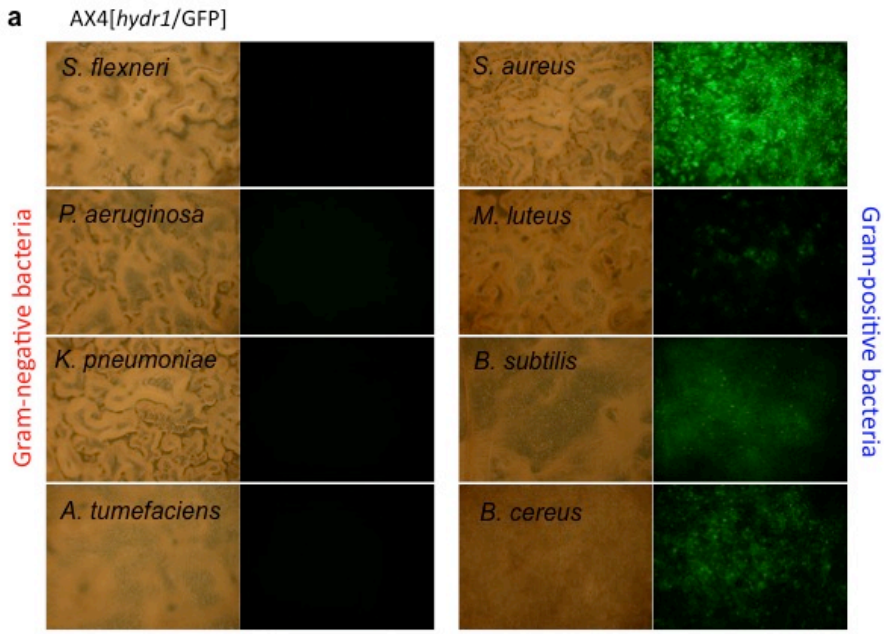
Bacterial discrimination by Dictyostelid amoebae reveals the complexity of ancient interspecies interactions

Waleed Nasser[1], Balaji Santhanam[2,3], Edward Roshan Miranda[2,4], Anup Parikh [2,3], Kavina Juneja[5], Gregor Rot[6], Chris Dinh[1], Rui Chen[2,3,4], Blaz Zupan[2,6], Gad Shaulsky[2,3,4] and Adam Kuspa[1,2,4*]

1 - Verna and Marrs McLean Department of Biochemistry and Molecular Biology, 2 – Department of Molecular and Human Genetics, 3 – Structural and Computational Biology and Molecular Biophysics Program, 4 – Developmental Biology Program, Baylor College of Medicine, Houston, TX 77030; 5 – Department of Biochemistry and Cell Biology, Rice University, Houston, TX 77005; 6 – Faculty of Computer and Information Science, University of Ljubljana, Slovenia.

* Corresponding author:

Adam Kuspa, Ph.D.
Department of Biochemistry and Molecular Biology
Baylor College of Medicine
One Baylor Plaza
Houston, TX 77030
Phone: 713-798-1060
E-mail: akuspa@bcm.edu


a
AX4[hydr1/GFP]
S. flexneri
P. aeruginosa
K. pneumoniae
A. tumefaciens
S. aureus
M. luteus
B. subtilis
B. cereus
Gram-negative bacteria
Gram-positive bacteria

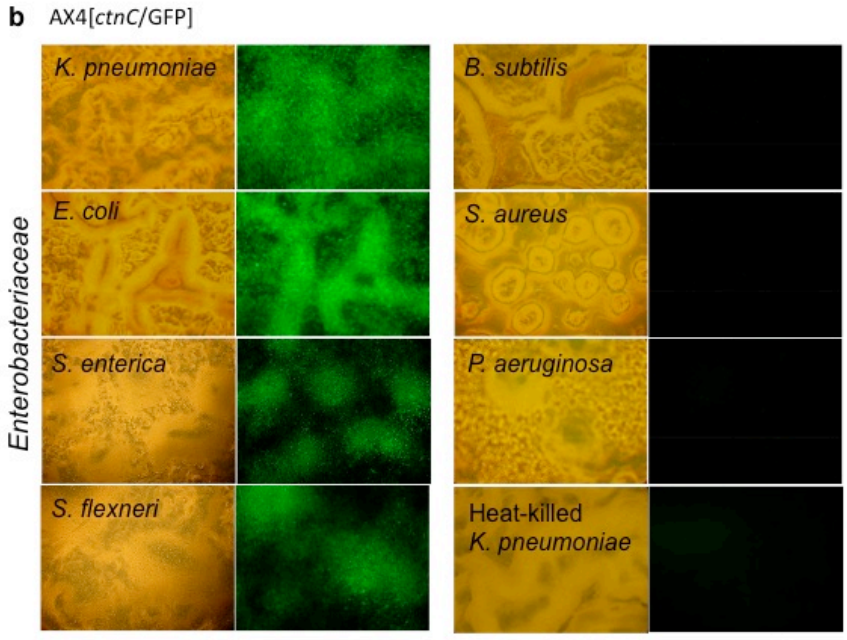
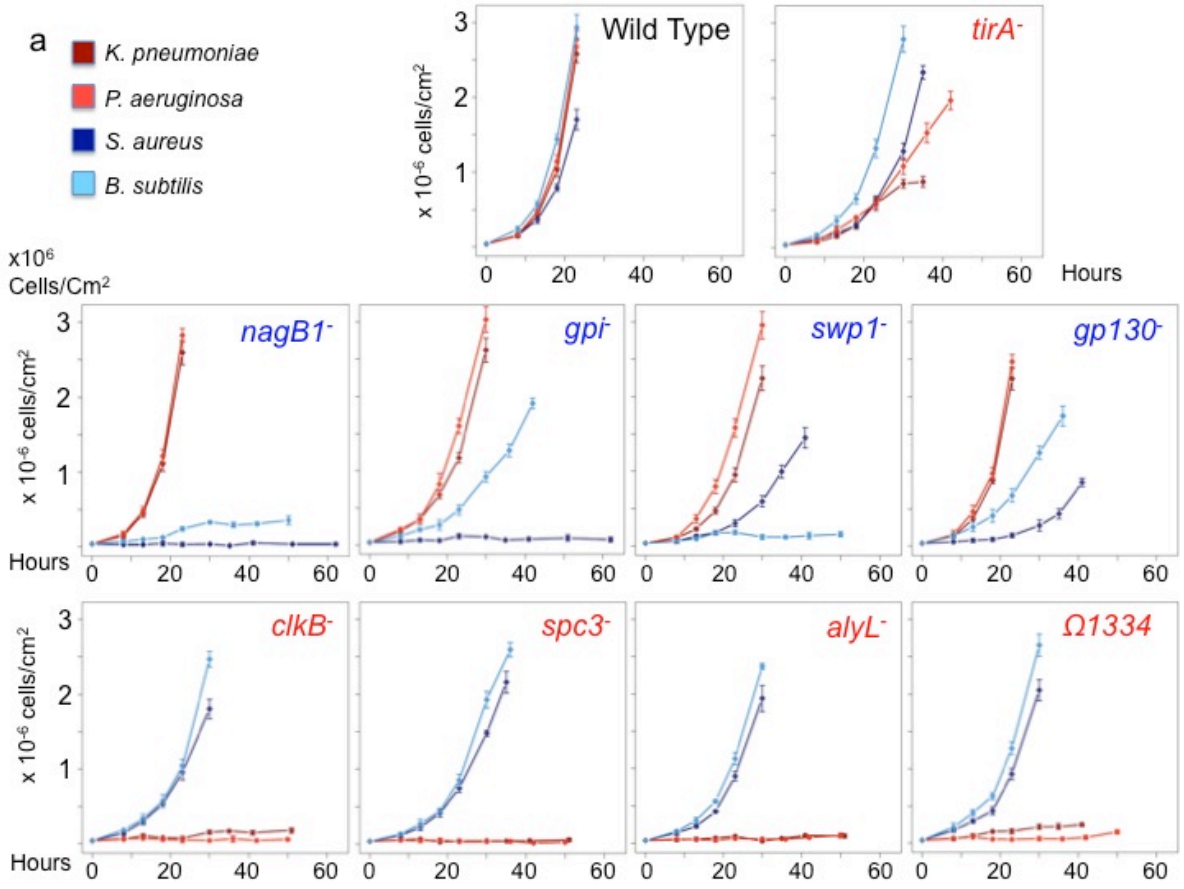
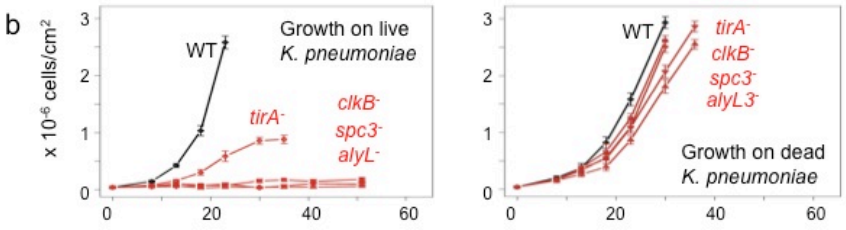

b
AX4[ctnC/GFP]
K. pneumoniae
E. coli
S. enterica
S. flexneri
B. subtilis
S. aureus
P. aeruginosa
Heat-killed
K. pneumoniae
Enterobacteriaceae

Figure S1. a, Wild-type (AX4) amoebae transformed with green fluorescent protein (GFP) coding sequence placed under the control of the *hydr1* (*DDB_G0293366*) promoter, a Gram-positive specific gene, AX4[*hydr1*/*GFP*], were mixed with different bacteria and spotted on buffered agar. The amoebae produce GFP and fluoresce when exposed to Gram-positive bacteria, but not when exposed to Gram-negative bacteria. Pictures depict bright-field and fluorescent microscopy using a 10X objective lens. **b**, Wild-type amoebae transformed with GFP placed under the control of *ctnC* promoter, AX4[*ctnC*/GFP], were mixed with different bacteria and spotted on buffered agar. The amoebae produce GFP and fluoresce when exposed to *Enterobacteriaceae* family bacteria, but not when exposed to heat-killed *Enterobacteriaceae*, or any other bacteria we tested.


a
K. pneumoniae
P. aeruginosa
S. aureus
B. subtilis
x10⁶ Cells/Cm²
x 10⁻⁶ cells/cm²
Hours
Wild Type
tirA⁻
nagB1⁻
gpi⁻
swp1⁻
gp130⁻
clkB⁻
spc3⁻
alyL⁻
Ω1334


b
x 10⁻⁶ cells/cm²
Growth on live K. pneumoniae
WT
tirA⁻
clkB⁻
spc3⁻
alyL⁻
Growth on dead K. pneumoniae
alyL3⁻

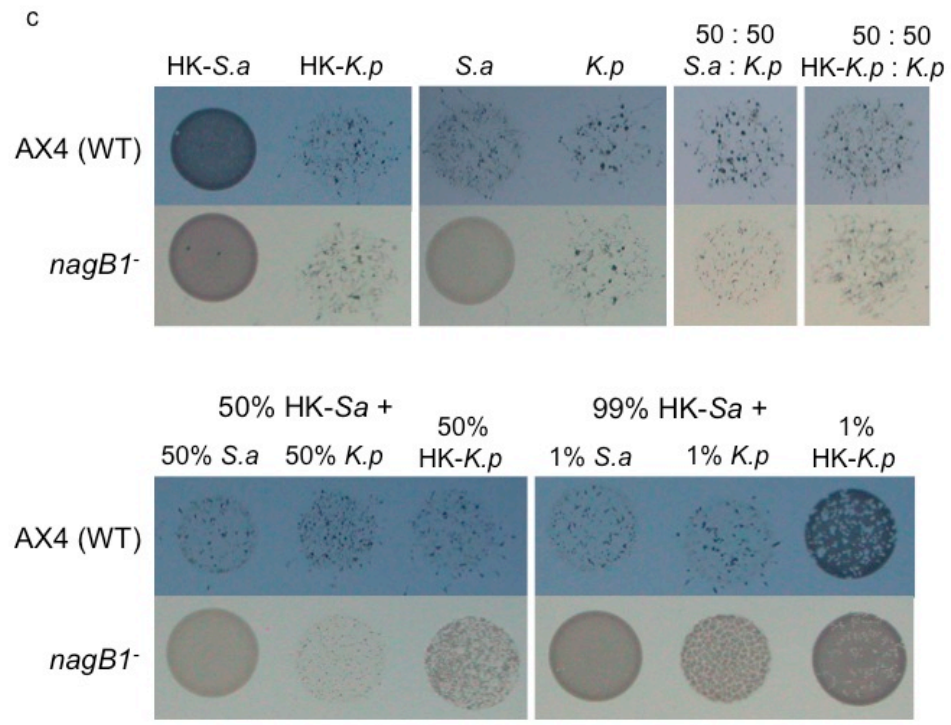


Figure S2. Growth of *D. discoideum* strains under different bacterial growth conditions. **a**, Growth curves of wild-type AX4 (WT) and mutant *D. discoideum* strains on four bacteria, *K. pneumoniae* , *P. aeruginosa*, *S. aureus*, and *B. subtilis*. *D. discoideum* strains (WT, *tirA*$^-$, *nagB1*$^-$, *gpi*$^-$, *swp1*$^-$, *gp130*$^-$, *clkB*$^-$, *spc3*$^-$, *alyL*$^-$, and Ω1334) were mixed with a thick bacterial culture and plated on buffered agar. *D. discoideum* growth was monitored by counting cells in a fixed surface area. **b**, Growth curve of WT and Gram(-) growth defective mutants on live bacteria (left) and heat-killed bacteria (right). WT and Gram(-) defective mutants exhibit similar growth phenotype on heat-killed Gram(-) bacteria. **c**, *D. discoideum* growth on heat-killed *S. aureus*. Rows represent wild type or *nagB1*$^-$ mutant amoeba as indicated on left, and columns represent different bacterial species or a mixture of bacterial species (heat-killed or live) in different mass ratios as indicated (HK, heat-killed; *S.a*, *S. aureus*; *K.p*, *K. pneumoniae*). Each spot is a co-culture of about 500 *D. discoideum* cells and a thick bacterial culture spotted on buffered agar and imaged after 4 days of incubation. The bacteria appear as tan areas within the spots. The dark speckles within the spots are a result of *Dictyostelium* aggregation and multicellular development following exhaustion of the bacterial food supply. Intermediate levels of feeding are indicated by partial clearing of the tan bacteria lawn. Similar results were obtained for the other Gram(+)-growth-defective mutants *gpi*$^-$ and *swp1*$^-$ (unpublished observations).

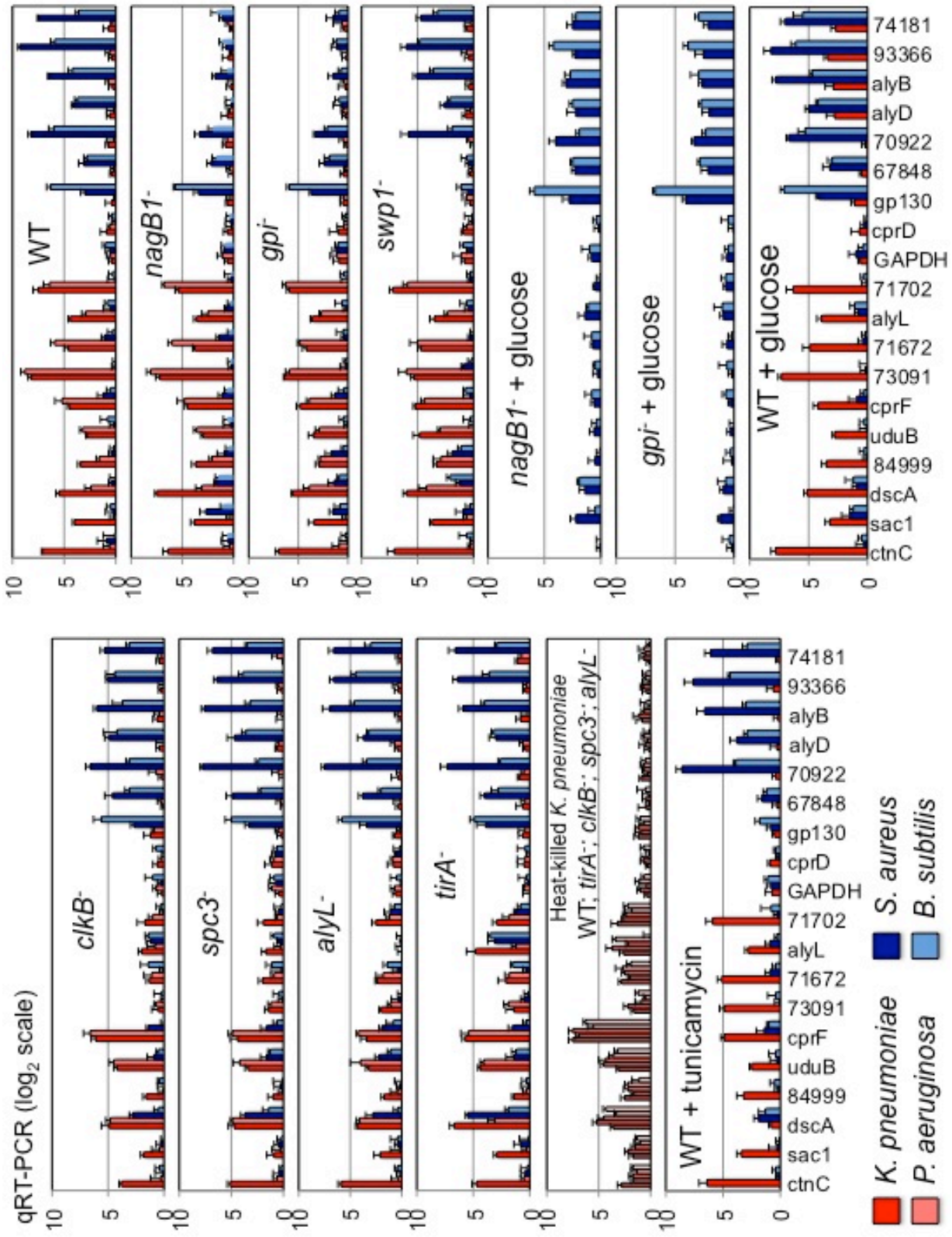

qRT-PCR (log2 scale)
clkB-
spc3-
alyL-
tirA-
Heat-killed K. pneumoniae
WT; tirA-; clkB-; spc3-; alyL-
WT + tunicamycin
WT
nagB1-
gpi-
swp1-
nagB1- + glucose
gpi- + glucose
WT + glucose
74181
93366
alyB
alyD
70922
67848
gp130
cprD
GAPDH
71702
alyL
71672
73091
cprF
uduB
84999
dscA
sac1
ctnC
K. pneumoniae
P. aeruginosa
S. aureus
B. subtilis

Figure S3. Expression (qRT-PCR) analysis of marker gene transcripts of *D. discoideum* strains under different bacterial growth conditions. *D. discoideum* strains WT, *nagB1*$^-$*, gpi*$^-$*, swp1*$^-$*, tirA*$^-$, *clkB*$^-$, *spc3*$^-$*,* and *alyL*$^-$ were mixed with *K. pneumoniae*, *P. aeruginosa*, *S. aureus*, and *B. subtilis*. WT amoebae were also mixed with *K. pneumoniae*, *S. aureus*, or *B. subtilis* in the presence of tunicamycin or glucose. The *nagB1*$^-$ and *gpi*$^-$ mutants were also mixed with *S. aureus*, and *B. subtilis* in the presence of glucose, and WT*, tirA*$^-$, *clkB*$^-$, *spc3*$^-$*,* and *alyL*$^-$ were mixed with Heat-killed *K. pneumoniae*. Relative mRNA levels were determined by qRT-PCR, using the gene specific primers (Supplemental Table S4) and normalizing them to the transcript levels of the histone H3a gene within each sample. Some of the genes are identified by the last five digits of their DDB_G number as annotated on dictyBase [1]. The full gene names can be reconstructed by adding the 5-digit number to the end of "DDB_G02".

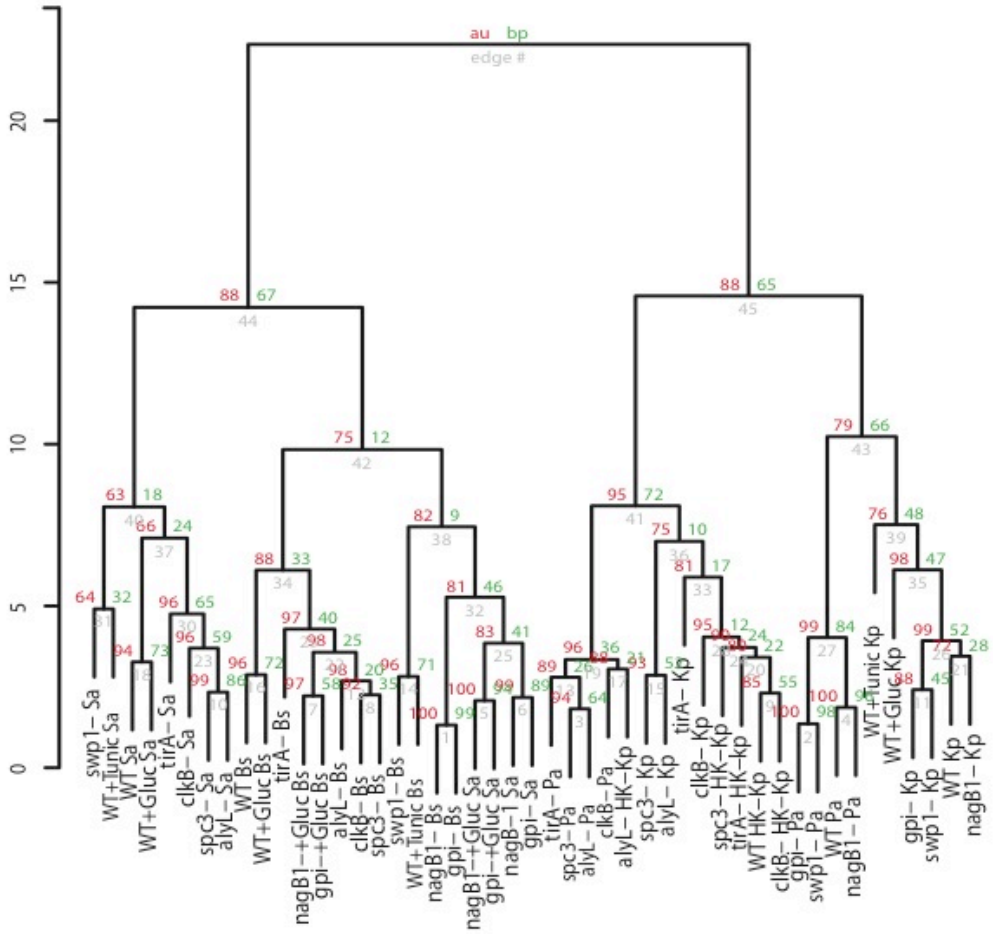


Figure S4. Hierarchical clustering of transcriptomes from *D. discoideum* strains under different bacterial growth conditions. Hierarchical clustering with multiscale bootstrap resampling (R package pvclust) of normalized mRNA levels of marker genes in wild-type (WT) or mutant (*nagB1*$^{-}$, *gpi*$^{-}$, *swp1*$^{-}$, *tirA*$^{-}$, *clkB*$^{-}$, *spc3*$^{-}$, *alyL*$^{-}$) *D. discoideum* strains grown on different bacterial species [*K. pneumoniae* (*K.p*), Heat-killed *K. pneumoniae* (HK-*K.p*), *P. aeruginosa* (*P.a*), *S. aureus* (*S.a*), or *B. subtilis* (*B.s*)]. Approximately unbiased *p*-values (au) and the bootstrap probabilities (bp) are shown for each branch, in red and green respectively [2]. The distances depicted are Euclidean distances with arbitrary units.

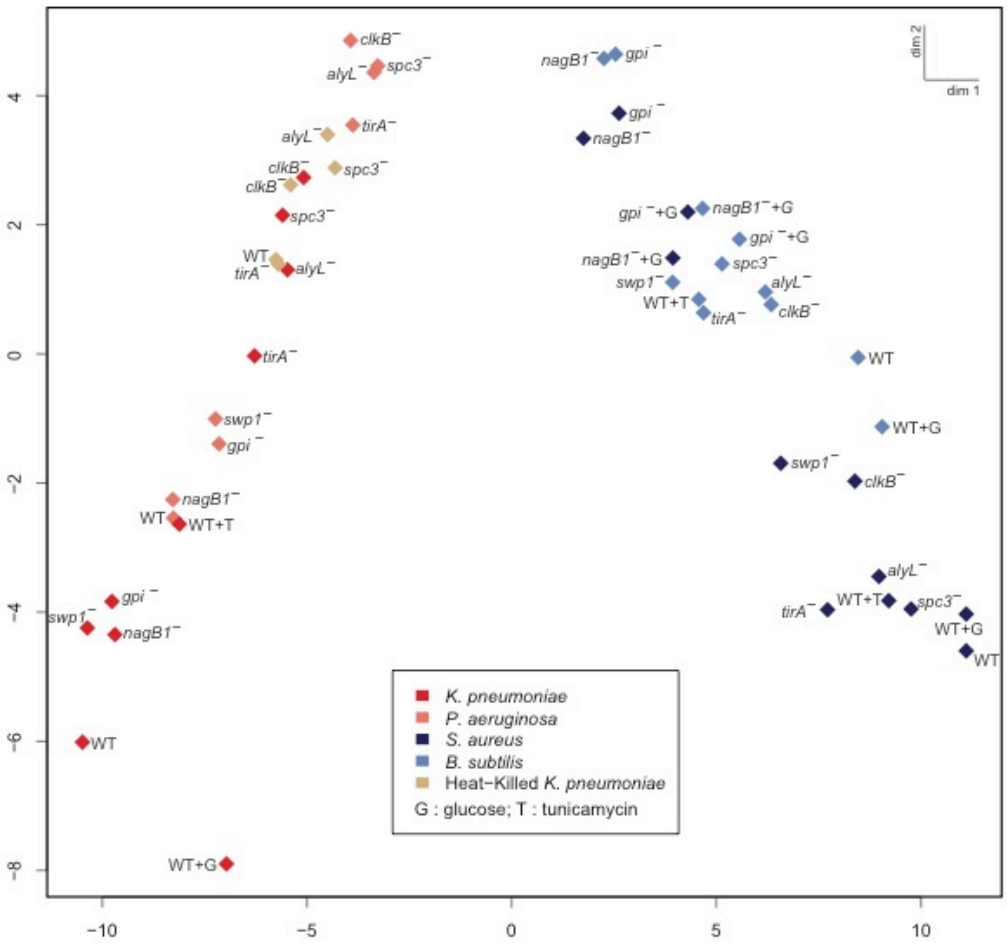


Figure S5. Multidimensional scaling analysis of transcriptomes from *D. discoideum* strains under different bacterial growth conditions. The same data set depicted in Figure S4 was subjected to classical multidimensional scaling (MDS) analysis (R function cmdscale) depicting the dissimilarities due to normalized mRNA levels of differentially expressed genes.

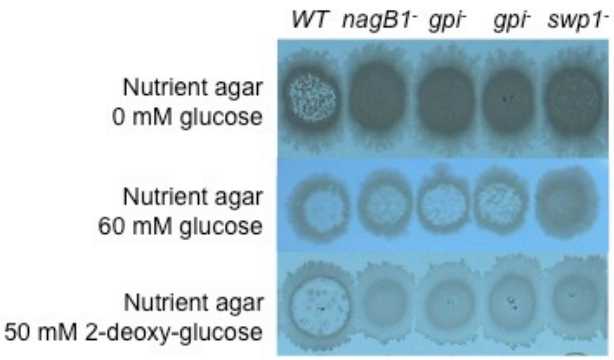


Figure S6. Glucose rescues *nagB1*⁻ and *gpi*⁻ mutants growth on *B. subtilis*.

Approximately 250 cells of AX4 wild-type (WT) *D. discoideum*, the *nagB1*⁻ mutant (AK1372), two different *gpi*⁻ mutant strains (AK1333 and AK1353), and the *swp1*- mutant (AK1321) mixed with an overnight culture of *B. subtilis* and spotted on Nutrient agar with no glucose added, 60 mM glucose, or 50 mM 2-deoxy-glucose. The images were acquired 4 days after plating. Glucose treatment rescued the growth phenotype of the *nagB1*⁻ and *gpi*⁻ mutants on *B. subtilis*, as indicated by the central clearing of the spots, but not the growth of the *swp1* mutant.

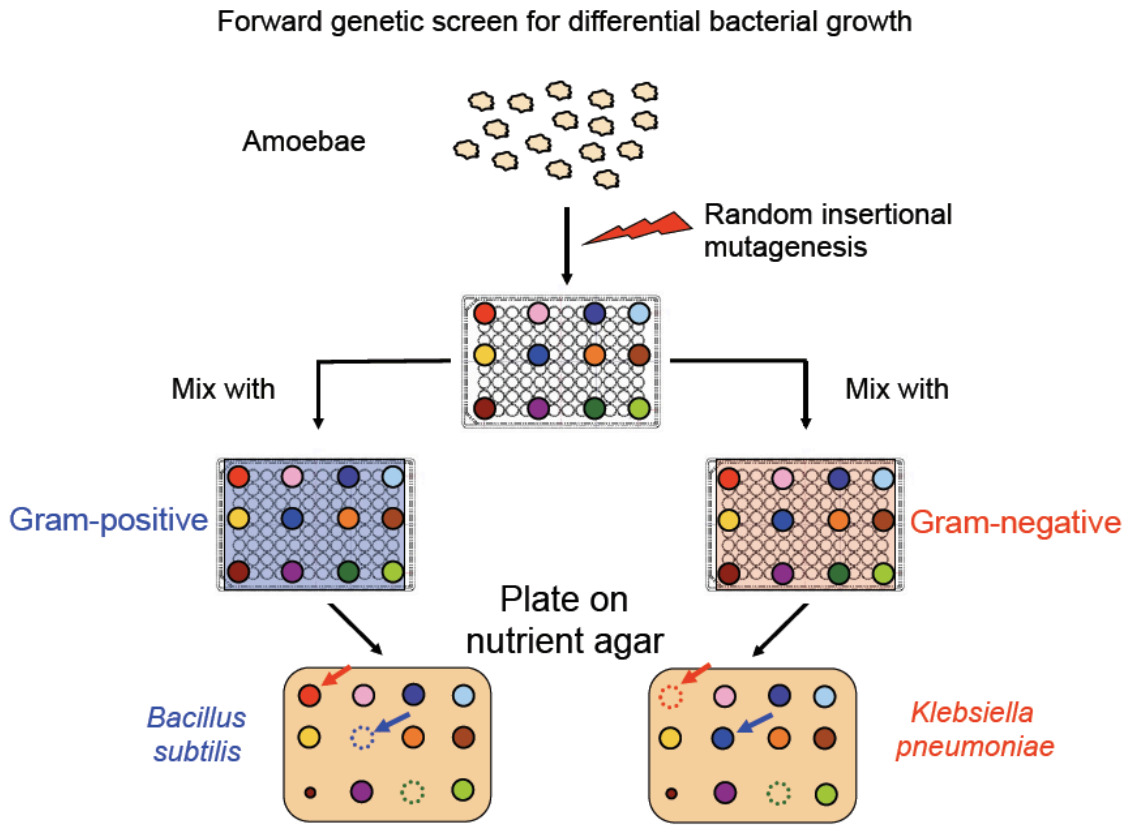


Figure S7. Scheme for the forward genetic screen to isolate mutants with a differential growth phenotype on bacteria. We subjected cultures of AX4 amoebae to plasmid insertional-mutagenesis using REMI [3] and plated for clonal growth in liquid media. Wells with transformed mutants colonies were consolidated into 96-well plates and the amoebae were allowed to grow to saturation. We mixed about 150-200 mutant cells with a slurry of Gram(+) *B. subtilis* bacteria and seperately with a slurry of Gram(-) *K. pneumoniae* bacteria. We spotted the amoebae-bacteria mixtures onto Nutrient media agar and monitored amoebal growth over five days. We picked mutants that displayed differential growth on the two bacteria (red or blue arrows), as well as those that showed slower or more rapid growth.

Supplemental Table S1 (separate file). An Excel spreadsheet containing the raw and processed RNAseq data used in this study.

Supplemental Table S2. *D. discoideum* strains used in this study.

Strain	Gene mutated	Growth Phenotype on Bacteria[1]	Annotation and Notes[2]	Reference
AX4	*axeA, axeB*	wild-type	Parental axenix strain for mutants generated in this study	[4]
1C7	*gp130*	Gram(+)-defective	gp130 adhesion protein	[5]
2F5	*gp130*	Gram(+)-defective	gp130 adhesion protein	[5]
AK1321	*swp1*	Gram(+)-defective	oligosaccharide transferase	This work
AK1333	*gpi*	Gram(+)-defective	glucose phosphate isomerase	This work
AK1353	*gpi*	Gram(+)-defective	glucose phosphate isomerase	This work
AK1372	*nagB1*	Gram(+)-defective	glucosamine-6-phosphate deaminase	This work
AK1336	*DDB_G0291279*	Gram(+)-defective	putative 11-transmembrane-domain transporter	This work
AK1339	*iliE-1*	Gram(+)-defective	lectin-like	This work
AK1334	unknown	Gram(−)-defective	insertion between two genes, DDB_G0295477/ DDB_G0271574, and upstream of putative noncoding-RNA gene	This work
AK1338	*clkB*	Gram(−)-defective	CDC7-related protein kinase DDB_G0278487	This work
AK1346	*spc3*	Gram(−)-defective	**s**ignal **p**eptidase **c**omplex subunit 3	This work
AK1350	*alyL*	Gram(−)-defective	amoeba lysozyme-like	This work
AK1303	*DDB_G0276559*	Gram(−)-defective	novel uncharacterized protein	This work
AK1319	*DDB_G0288161*	Fast growth	novel uncharacterized protein	This work
AK1320	*DDB_G0281785*	Fast growth	B-box zinc finger binding, and FNIP repeats	This work
AK1302	*rbsK*	No growth	D-ribose metabolism	This work
AK1301	*DDB_G0277169*	Slow growth	novel uncharacterized protein	This work
AK1304	*pakB*	Slow growth	phagocytosis regulator	This work
AK1305	*DDB_G0292196*	Slow growth	novel uncharacterized protein	This work
AK1306	*DDB_G0270300*	Slow growth	novel uncharacterized protein	This work
AK1307	*DDB_G0284227*	Slow growth	nucleic acid binding and nuclease activity	This work
AK1309	*DDB_G0277667*	Slow growth	cyclin domain containing protein	This work
AK1311	*pelo*	Slow growth	translation factor	This work
AK1313	*DDB_G0293440*	Slow growth	methyl transferase	This work
AK1315	*DDB_G0281967*	Slow growth	novel uncharacterized cell surface protein	This work
AK1316	*DDB_G0288459*	Slow growth	transglutaminase-like protein	This work
AK1327	*shkA*	Slow growth	**SH**2 domain-containing **k**inase	This work
AK1331	*arpF*	Slow growth	**a**ctin **r**elated **p**rotein 6	This work
AK1332	*DDB_G0281447*	Slow growth	2 transmembrane domains	This work
AK1335	*DDB_G0285803*	Slow growth	Putative acetyltransferase	This work
AK1337	*dhcA*	Slow growth	cytoplasmic **d**ynein **h**eavy chain	This work
AK1340	*DDB_G0269608*	Slow growth	Novel uncharacterized protein	This work
AK1341	*DDB_G0276277*	Slow growth	Putative oxidoreductase	This work

AK1342	*DDB_G0272382*	Slow growth	PH and Phox domains	This work
AK1343	*DDB_G0292054*	Slow growth	Carbohydrate-binding	This work
AK1345	*DDB_G0293476*	Slow growth	Thrombospondin type 3 repeat	This work
AK1348	*qtrt1*	Slow growth	**Q**ueuine **tR**NA-ribosyl**T**ransferase **1**	This work
AK1371	*empB*	Slow growth	component of COPI-coated vesicles	This work
AK1373	*elp4*	Slow growth	RNA polymerase II elongation complex	This work
AK1374	*cct3*	Slow growth	**C**haperonin **C**ontaining **T**CP1 subunit **3**	This work
AK1362	*DDB_G0279939*	Slow growth	Novel uncharacterized protein	This work

Table S3. Bacterial strains used in this study.

Bacterial species (isolate)	**Phylum, Class, Order**	**Source**
Klebsiella pneumoniae (Environmental)	*Proteobacteria, Gamma Proteobacteria, Enterobacteriales*	Kuspa Lab (Baylor College of Medicine)
Bacillus subtilis *AG1431* (Environmental)	*Firmicutes, Bacilli, Bacillales*	Jue D. Wang (University of Wisconsin, Madison WI)
Pseudomonas aeruginosa *H45006* (Clinical)	*Proteobacteria, Gamma Proteobacteria, Pseudomonadales*	James Versalovic (Baylor College of Medicine, Houston TX)
Staphylococcus aureus *ATCC 49230* (Clinical)	*Firmicutes, Bacilli, Bacillales*	Heidi Kaplan (University of Texas, Houston TX)
Pseudomonas fluorescens *WC5365* (Environmental)	*Proteobacteria, Gamma Proteobacteria, Pseudomonadales*	Elizabeth Ostrowski (University of Houston, Houston TX)
Pseudomonas fluorescens *f-01* (Environmental)	*Proteobacteria, Gamma Proteobacteria, Pseudomonadales*	Elizabeth Ostrowski (University of Houston, Houston TX)
Pseudomonas fluorescens *f-05* (Environmental)	*Proteobacteria, Gamma Proteobacteria, Pseudomonadales*	Elizabeth Ostrowski (University of Houston, Houston TX)
Pseudomonas stutzeri *W47237-W1* (Clinical)	*Proteobacteria, Gamma Proteobacteria, Pseudomonadales*	James Versalovic (Baylor College of Medicine, Houston TX)
Bacillus cereus *ATCC 10987* (Clinical)	*Firmicutes, Bacilli, Bacillales*	Anthony Maresso (Baylor College of Medicine, Houston TX)
Bacillus anthracis *sterne* (Environmental)	*Firmicutes, Bacilli, Bacillales*	Anthony Maresso (Baylor College of Medicine, Houston TX)
Agrobacterim tumefaciens *A348* (Environmental)	*Proteobacteria, Alphaproteobacteria, Rhizobiales*	Heidi Kaplan (University of Texas, Houston)
Escherichia coli *B rel 606* (Environmental)	*Proteobacteria, Gamma Proteobacteria, Enterobacteriales*	Elizabeth Ostrowski (University of Houston, Houston TX)
Salmonella enterica *ATCC 14028* (Clinical)	*Proteobacteria, Gamma Proteobacteria, Enterobacteriales*	Herbert DuPont (University of Texas, Houston)
Shigella flexneri *ATCC 12022* (Clinical)	*Proteobacteria, Gamma Proteobacteria, Enterobacteriales*	Herbert DuPont (University of Texas, Houston)
Micrococcus luteus (Environmental)	*Actinobacteria, Actinobacteridae, Actinomycetales*	Peter Newell (deposited at dictyBase stock center)
Enterococcus faecalis *OGRIF* (Clinical)	*Firmicutes, Bacilli, Lactobacillales*	Danielle Garsin (University of Texas, Houston)

Bacterial species were classified according to their taxonomy (NCBI taxonomy website).

Supplement Table S4. Primers used for qRT-PCR transcriptional profile analysis.

DDB_G number	Gene name	Forward primer (5'-3')	Reverse primer (5'-3')
0267402	*H3a*	GGTTCTAAACAAGCCCATAAACA	CTCTAAGAGCGACAGTAC
0275153	*gpdA*	GGTTGTCCCAATTGGTATTAATGG	CCGTGGGTTGAATCATATTTGAAC
0278721	*cprD*	TCAAACCTCTGCTTCTGGTCA	AGAAGCCATTGTTGCGATACCA
0276479	*ctnC*	TCAAAAGTTATGTCATCAAGCGAAGAATG	TCCATAAAACCATTAATTCCAACTGCATC
0268848	*DDB_G0268848*	TCACTAGTCTCAACTCCATCAAC	AGATTTATCAAAACTAGTGACATCAGTTTCAG
0273063	*dscA-1*	TCCACACTCAATTTAATTCTGCCT	TGTATGATGTAACCCATTGATCAG
0284999	*DDB_G0284999*	AGAATGCTGAAAAGTTCACTGAAGCT	TGGAGCGATTTCTTGTAATGCTTC
0268600	*uduB*	ACCAGCAACCTCTGATCCA	TCTGTTGGCTGGTTCAGAG
0279185	*cprF*	TCAATCTCAAGGTTCCAATAGTTTCAC	TGTAACCATTAATACCCCAGTCAAG
0273091	*DDB_G0273091*	AGAACATGATCATGACCATGAAGAAG	TGAGTGGCAACAATTGGAATAACTG
0271672	*DDB_G0271672*	TGTCACGCTCCAAGTGGT	TGATGGAATGCTAATGCAACTAATAAAGCT
0286229	*alyL*	ACCAAGTTTTCGCTACTTGTGTTAG	AGAGATCCTCGCAAACTTTTGTTG
0271702	*DDB_G0271702*	TCTCTGGTCCAGGTTGTTC	ACTGAAACTTTTTGAGAGTCTGGAAG
0279921	*gp130*	TGGTAGTATACCAGAAGGGTATTG	AGTTTCACAATTGGTTGGTAATGATTTTTC
0267848	*DDB_G0267848*	GGTCTTATGGATACTGTAACGGT	TGTTCAACAGTGATAAGACCAGTTG
0270922	*DDB_G0270922*	TGTTTCTGGGATGGTAAGGTTTG	TCATTGTGGATTGATGAACAATGGACT
0275119	*alyD-1*	AGGTTCTGCAGGTACCTCA	ACAAACACCATAGAAACCTCCAGT
0275119	*alyB*	AGTTCCATCATGCAGTGGTGCT	AGTGAGAGAAGTAACAGTGGCA
0293366	*hydr1 DDB_G0293366*	ACGCAACTTGTGGTTGTCCA	ACCACTACATGCGGTACCT
0274181	*hydr2 DDB_G0274181*	TGCTCATATCGGTGTTTACACATC	ACAGCTGTCACCAGTGTC

References

1. dictybase.org (2012).
2. Suzuki, R., and Shimodaira, H. (2006). pvclust: Hierarchical Clustering with P-Values via Multiscale Bootstrap Resampling.
3. Kuspa, A., and Loomis, W.F. (1992). Tagging developmental genes in Dictyostelium by restriction enzyme-mediated integration of plasmid DNA. Proc. Natl. Acad. Sci. USA *89*, 8803-8807.
4. Knecht, D.A., Cohen, S.M., Loomis, W.F., and Lodish, H.F. (1986). Developmental regulation of Dictyostelium discoideum actin gene fusions carried on low-copy and high-copy transformation vectors. Mol. Cell. Biol. *6*, 3973-3983.
5. Chia, C.P., Gomathinayagam, S., Schmaltz, R.J., and Smoyer, L.K. (2005). Glycoprotein gp130 of dictyostelium discoideum influences macropinocytosis and adhesion. Mol Biol Cell *16*, 2681-2693.