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Figures and figure supplements

Kin cell lysis is a danger signal that activates antibacterial pathways of *Pseudomonas aeruginosa*

Michele LeRoux, et al.

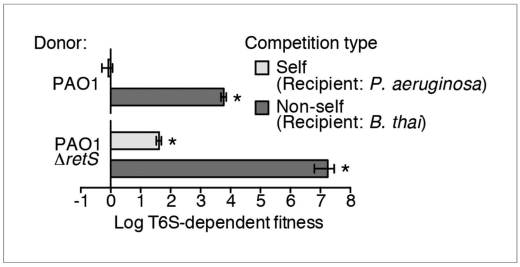

Figure 1. Wild-type *P. aeruginosa* cells display a strong T6S-dependent fitness advantage in co-culture with non-self but not self competitors. Outcome of growth competition experiments measuring fitness of *P. aeruginosa* PAO1 parental or Δ*retS* strains in co-cultures with self or non-self recipients under T6SS-promoting conditions. The self recipient was *P. aeruginosa* Δ*tse1-4* Δ*tsi1-4* in the strain background corresponding to the donor genotype (PAO1 or PAO1 Δ*retS*). T6S-dependent fitness was parental donor competitive index (change [final/initial] in ratio of donor and recipient colony forming units [c.f.u.]) normalized to Δ*tssM1* competitive index. Error bars represent ±standard deviation (SD); n = 3 co-cultures. Asterisks denote a fitness advantage significantly >1 ($p < 0.01$).

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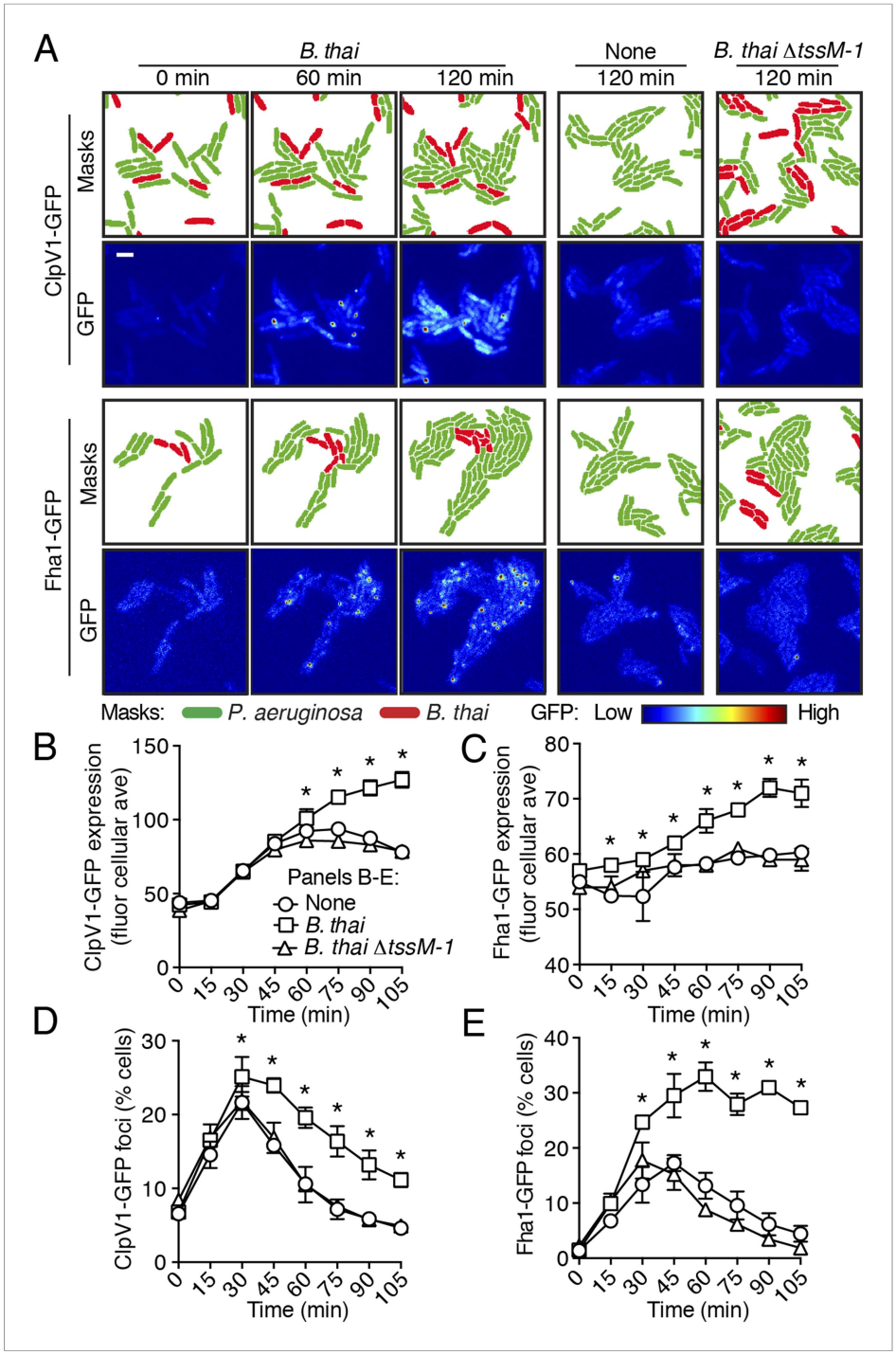

Figure 2. Non-self competitor bacteria stimulate expression and activity of the H1-T6SS. (**A**) H1-T6SS expression is increased in *P. aeruginosa* co-cultured with *B. thai* expressing an active T6SS. Time-lapse fluorescence microscopy (TLFM) sequences of *P. aeruginosa clpV1-gfp* (upper) or *fha1-gfp* (lower) in monoculture or in co-culture with the indicated competitor. Cropped regions from representative time-points are displayed. Remaining time points for monoculture and co-culture with *B. thai* Δ*tssM-1* are depicted in ***Figure 2—figure supplement 1***; see also ***Videos 1 and 2***. Masks colored by cell identity depict automated cell identification generated from the phase image. Scale bar, 6 μm. (**B**–**C**) Quantification of H1-T6SS expression from the *P. aeruginosa clpV1-gfp* (**B**) and *fha1-gfp* (**C**) mono and co-culture TLFM experiments described in (**A**). Average cellular GFP intensity for *P. aeruginosa* cells was calculated from background-subtracted images. (**D**–**E**) H1-T6SS activity is increased in the presence of *B. thai* with an active T6SS. Percentage of *P. aeruginosa clpV1-gfp* (**D**) or *fha1-gfp* (**E**) cells with GFP foci for experiments described

Figure 2. continued on next page

Figure 2. Continued

in (**A**). Error bars represent ±SD; n = 3 fields. Asterisks indicate significant differences when *B. thai* was present ($p < 0.05$).

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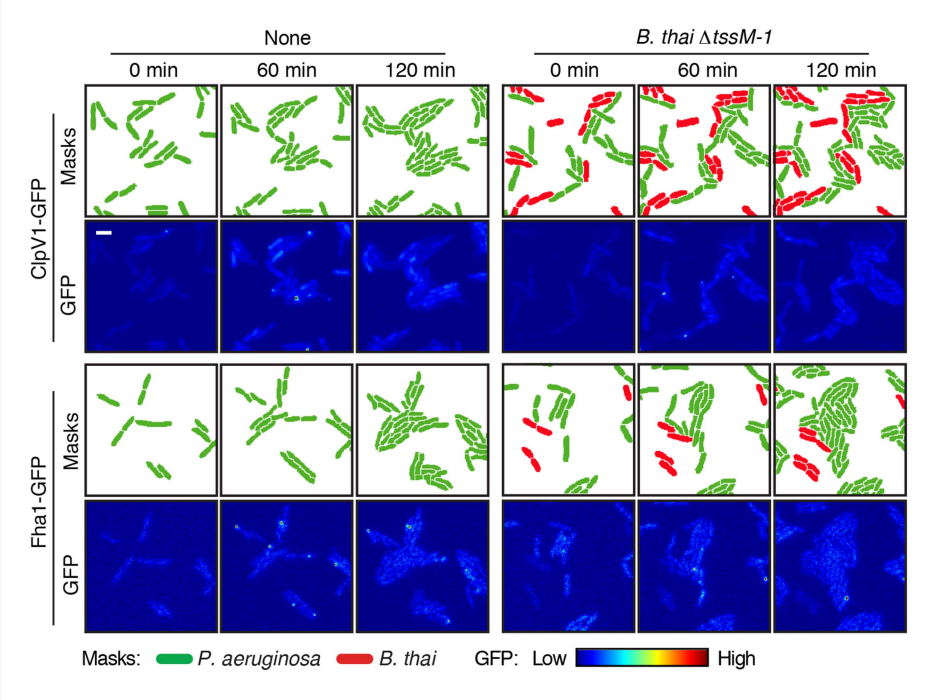

Figure 2—figure supplement 1. Competitors require an active T6SS to stimulate the *P. aeruginosa* H1-T6SS. TLFM sequences of *P. aeruginosa clpV1-gfp* (upper) and *fha1-gfp* (lower) cultivated either without competitor or with *B. thai* Δ*tssM-1* as depicted in ***Figure 2A***. Scale bar, 6 μm.

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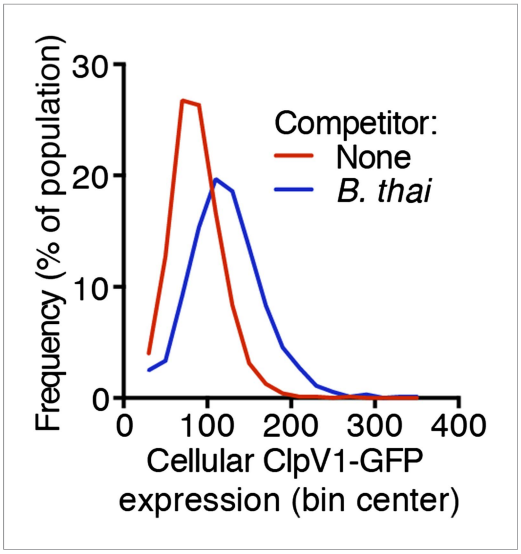

Figure 2—figure supplement 2. Increased H1-T6SS expression occurs throughout the population. Histograms of cellular ClpV1-GFP intensity of *P. aeruginosa clpV1-gfp* following 90 min of growth in monoculture or in co-culture with *B. thai*. Histograms bin size is 20 intensity units and is normalized to total cells.
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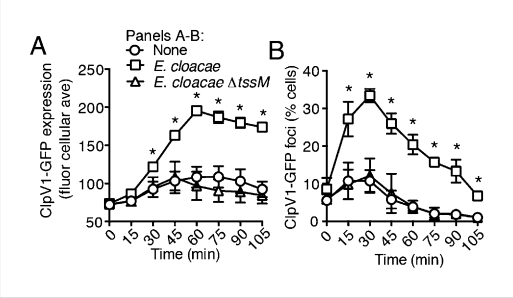

Figure 2—figure supplement 3. *E. cloacae* stimulates the H1-T6SS of *P. aeruginosa* in a T6S-dependent manner. Average cellular ClpV1-GFP expression (**A**) and the percentage of cells with ClpV1-GFP foci (**B**) of *P. aeruginosa clpV1-gfp* in monoculture or in co-culture with the indicated *E. cloacae* competitor. Data was collected and analyzed as described in ***Figure 2***. Error bars represent ±SD; n = 3 fields. Asterisks indicate significant differences when *B. thai* was present ($p < 0.05$). See also ***Video 3***.
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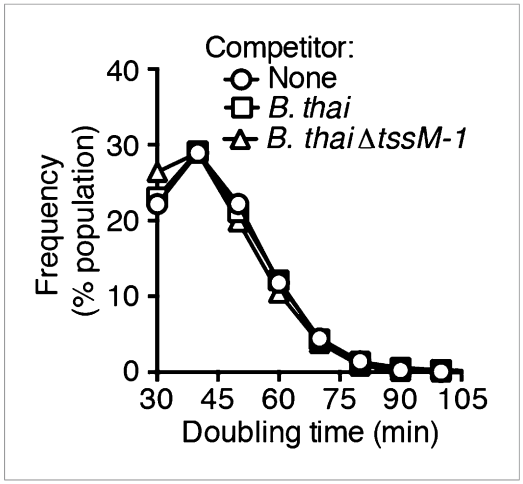

Figure 2—figure supplement 4. *P. aeruginosa* doubling time is not affected by the presence of *B. thai*. Histograms depicting *P. aeruginosa* doubling times during growth in monoculture or co-culture with *B. thai* under TLFM conditions. Error bars represent ±SD; n = 3 fields.

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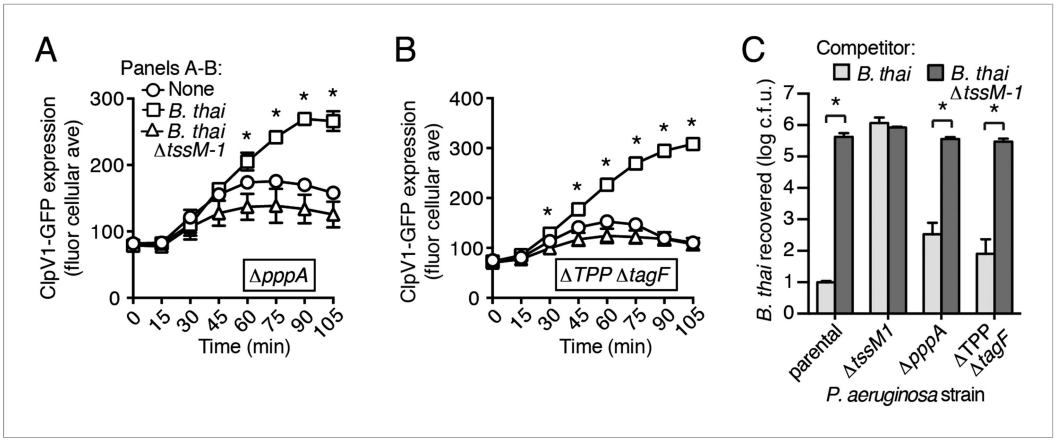

Figure 3. PARA does not require the TPP. (**A**–**B**) Increased H1-T6SS expression in the presence of *B. thai* does not require a functional TPP. Average ClpV1-GFP cellular fluorescence intensity in *P. aeruginosa clpV1-gfp* Δ*pppA* (**A**) and ΔTPP Δ*tagF* (**B**) backgrounds during monoculture or co-culture with the indicated competitors. Error bars represent ±SD; n = 3 fields. Asterisks indicate significant differences when *B. thai* was present ($p < 0.05$). Corresponding H1-T6SS activity is shown in ***Figure 3—figure supplement 1***. (**C**) The TPP is not required for preferential targeting of *B. thai* with an active T6SS. Outcome of growth competition experiments measuring survival of *B. thai* following co-culture with the indicated *P. aeruginosa* strain under T6SS-promoting conditions. Error bars represent ±SD; n = 3 co-cultures.

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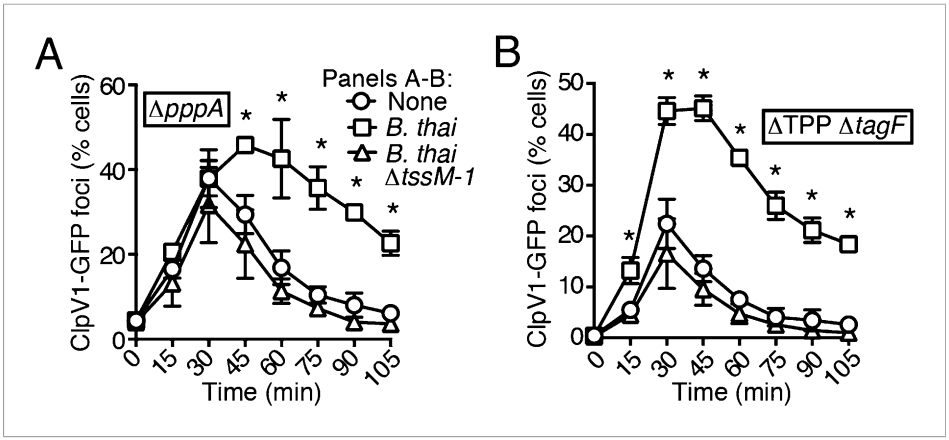

Figure 3—figure supplement 1. Elevated H1-T6SS activity in the presence of *B. thai* does not require the TPP. Percentage of cells containing ClpV1-GFP foci from *P. aeruginosa clpV1-gfp* strains of Δ*pppA* (**A**) or ΔTPP Δ*tagF* (**B**) backgrounds in monoculture or in co-culture with the indicated competitor. Error bars represent ±SD; n = 3 co-cultures. Asterisks indicate significant differences when *B. thai* was present ($p < 0.05$).

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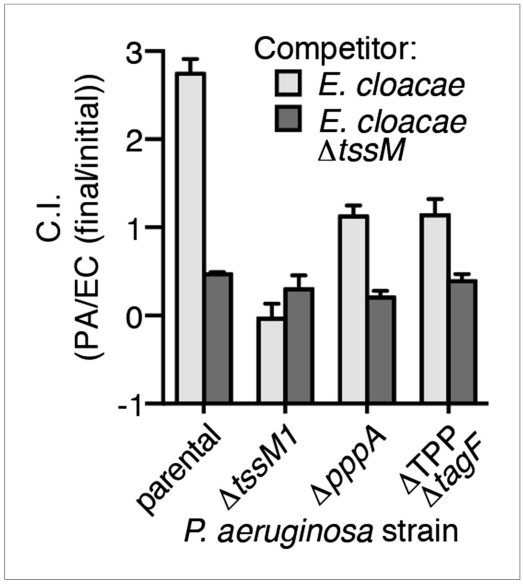

Figure 3—figure supplement 2. *P. aeruginosa* does not require the TPP to differentially target *E. cloacae* with a T6SS. Outcome of interspecies growth competition experiments measuring competitive index (C.I.) of *P. aeruginosa–E. cloacae* co-cultures grown under T6SS-promoting conditions. PA, *P. aeruginosa*; EC, *E. cloacae*. Error bars represent ±SD; n = 3 co-cultures.
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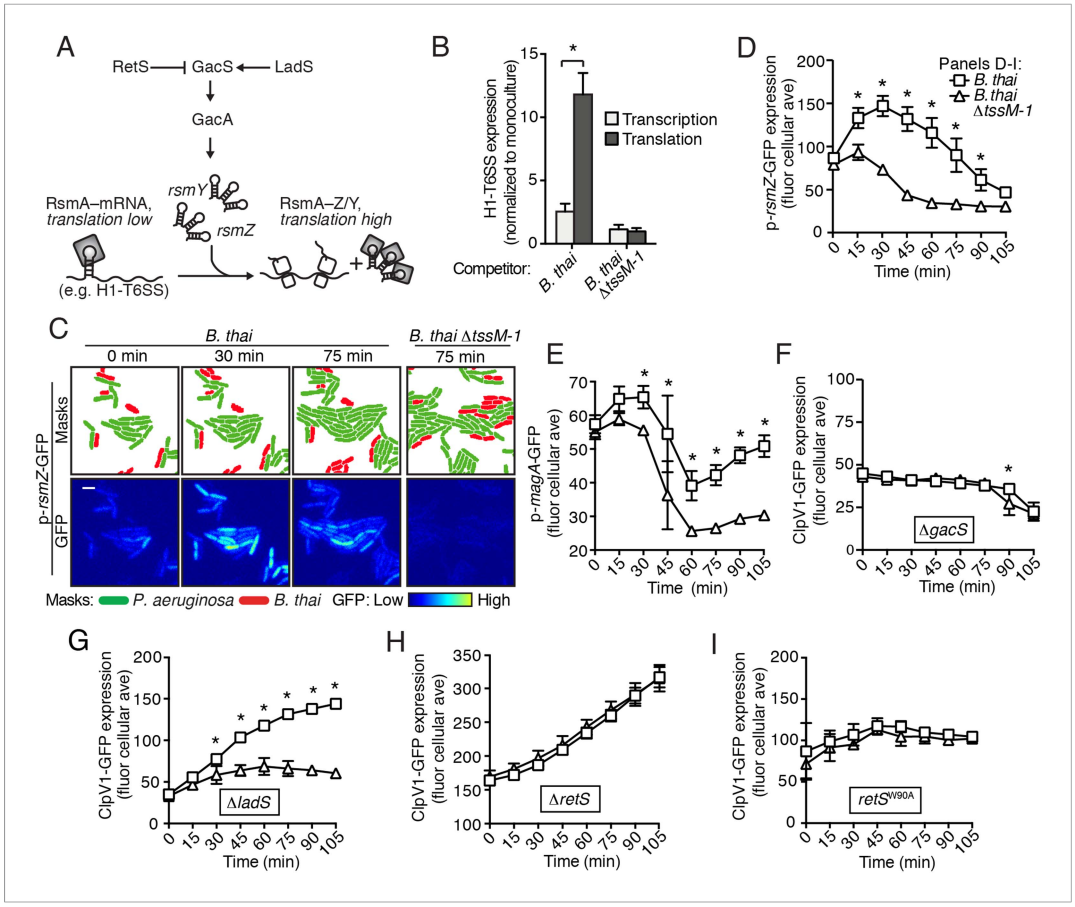

Figure 4. The Gac/Rsm pathway is required for PARA. (**A**) Schematic depicting the Gac/Rsm pathway of *P. aeruginosa*. The orphan sensor kinases RetS and LadS exert opposing activity on a third sensor kinase, GacS, which in turn activates its cognate response regulator, GacA. Once active, GacA promotes increased transcription of the small RNAs *rsmY* and *rsmZ*. These molecules bind and sequester RsmA; therefore, when abundant, they prevent RsmA binding and destabilization of target mRNAs, including H1-T6SS transcripts. (**B**) Elevated H1-T6SS expression in the presence of *B. thai* occurs primarily at the post-transcriptional level. *P. aeruginosa* strains bearing chromosomally encoded transcriptional or translational fusions to *tssA1* (***Brencic and Lory, 2009***) were incubated with the indicated competitor. Fold H1-T6SS increase in expression was determined by normalizing *P. aeruginosa* co-cultures to the corresponding strain cultivated in monoculture. n = 3 co-cultures; asterisk indicates significant differences between translational and transcriptional activity ($p < 0.05$). (**C**) *RsmZ* expression is elevated in the presence of *B. thai* containing a T6SS. Cells masks and the GFP fluorescence channel from representative TLFM sequences of the indicated *P. aeruginosa* p-*rsmZ-gfp* co-cultures. Additional time points are shown in ***Figure 4—figure supplement 1***; see also ***Video 4***. (**D**) Average cellular fluorescence intensity from *P. aeruginosa* of p-*rsmZ-gfp* corresponding to (**C**). (**E**) Expression of MagA is elevated in the presence of $T6S^{BT}$. Average cellular GFP intensity for *P. aeruginosa* p-*magA-gfp* in co-culture with *B. thai* or *B. thai* Δ*tssM-1*. (**F**) GacS is required for H1-T6SS activation in response to $T6S^{BT}$. ClpV1-GFP expression was quantified for co-cultures of *P. aeruginosa* Δ*gacS clpV1-gfp* with the indicated *B. thai* competitor. (**G**) LadS is not required for elevated H1-T6SS expression in the presence of *B. thai*. Average cellular ClpV1-GFP intensity of *P. aeruginosa* Δ*ladS clpV1-gfp* in co-culture with the indicated competitor. See ***Figure 4—figure supplement 2A*** for H1-T6SS activity. (**H**) *P. aeruginosa* cells lacking *retS* are unable to respond to $T6S^{BT}$. Average cellular ClpV1-GFP intensity of *P. aeruginosa* Δ*retS clpV1-gfp* in co-culture with the indicated competitor. See ***Figure 4—figure supplement 2B*** for H1-T6SS activity. (**I**) A conserved residue in the periplasmic domain of RetS is required for *P. aeruginosa* response to $T6S^{BT}$. Average cellular ClpV1-GFP intensity of *P. aeruginosa clpV1-gfp* $retS^{W90A}$ cultivated in the presence of the indicated competitor. See ***Figure 4—figure supplement 2C*** for H1-T6SS activity. n = 3 fields; asterisks indicate significant differences when *B. thai* was present ($p < 0.05$).

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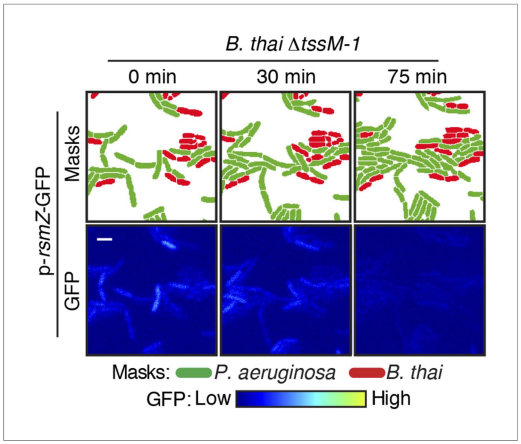

Figure 4—figure supplement 1. *RsmZ* expression is not stimulated by *B. thai* lacking an active T6SS. Representative TLFM sequences of *P. aeruginosa* p-*rsmZ-gfp* cultivated in co-culture with *B. thai* Δ*tssM-1*.

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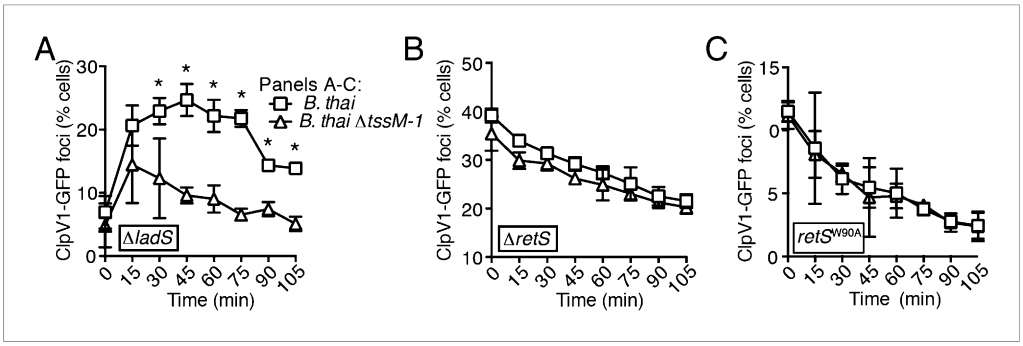

Figure 4—figure supplement 2. PARA-associated increases in H1-T6SS activity depend on RetS but not LadS. (**A**) LadS is not required for increased activity of the H1-T6SS in the presence of *B. thai*. Percentage of *P. aeruginosa clpV1-gfp* Δ*ladS* cells with fluorescent foci during co-culture with the indicated *B. thai* strain. (**B**) Stimulation of H1-T6SS activity by *B. thai* requires RetS. Percentage of *P. aeruginosa clpV1-gfp* Δ*retS* cells with fluorescent foci during co-culture with the indicated *B. thai* strain. (**C**) A putative signal-binding RetS mutant does not respond to the presence of *B. thai*. Percentage of *P. aeruginosa clpV1-gfp retS*^{W90A} cells with fluorescent foci during co-culture with the indicated *B. thai* strain. Error bars represent ±SD; n = 3 co-cultures. Asterisks indicate significant differences when *B. thai* was present ($p < 0.05$).

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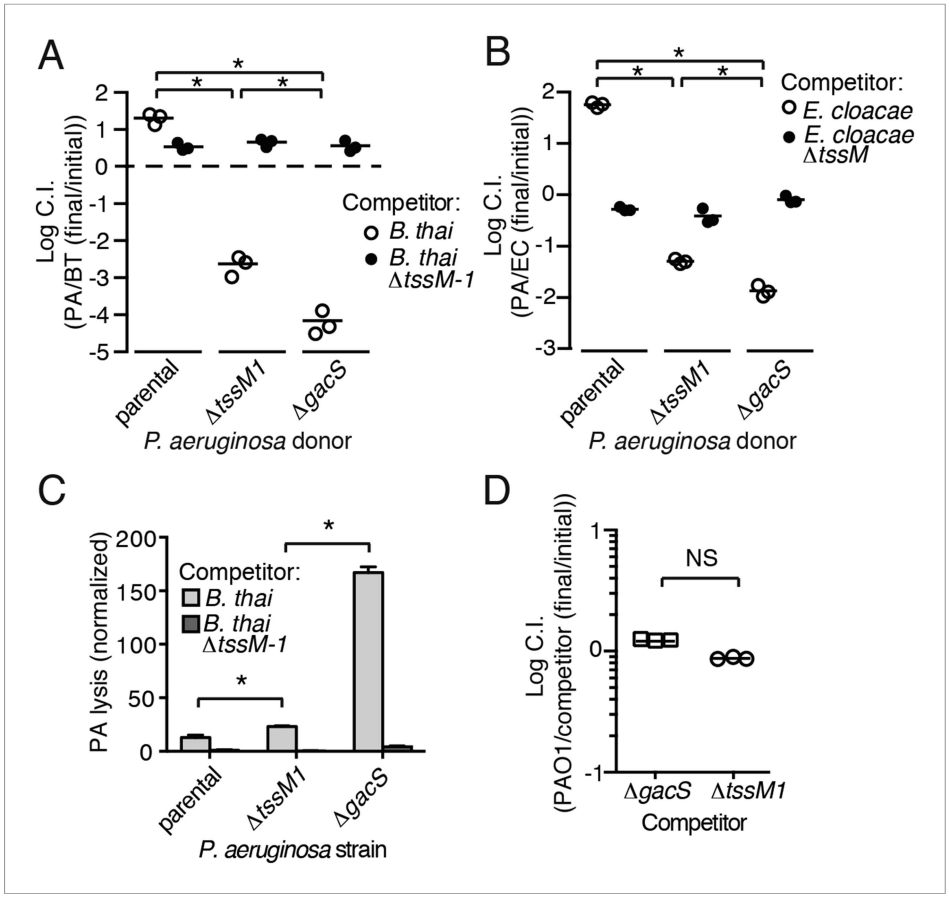

Figure 5. Disruption of the Gac/Rsm pathway results in a profound fitness defect in interspecies co-culture. (**A**–**B**) A *P. aeruginosa* strain with an inactivated Gac/Rsm pathway displays fitness defects beyond a strain lacking H1-T6S. Outcome of interspecies growth competition experiments between the indicated *P. aeruginosa* and *B. thai* (**A**) or *E. cloacae* (**B**) strains. n = 3 co-cultures. C.I., competitive index. PA, *P. aeruginosa*. BT, *B. thai*. EC, *E. cloacae*. (**C**) *P. aeruginosa* lysis promoted by $T6S^{BT}$ is increased in a strain lacking a functional Gac/Rsm pathway. *P. aeruginosa* lysis events from TLFM sequences were normalized to initial number of contacts with *B. thai*. See also ***Video 5***. n = 3 fields. (**D**) A *gacS* deletion strain does not alter growth rate. Outcome of intraspecies growth competition experiments between PAO1 and the indicated competitor strains under conditions identical to those used in (**A**–**B**). n = 3 co-cultures. (**A**–**D**) Error bars represent ±SD; asterisks indicate significant differences between indicated groups ($p < 0.05$). NS, not significant.

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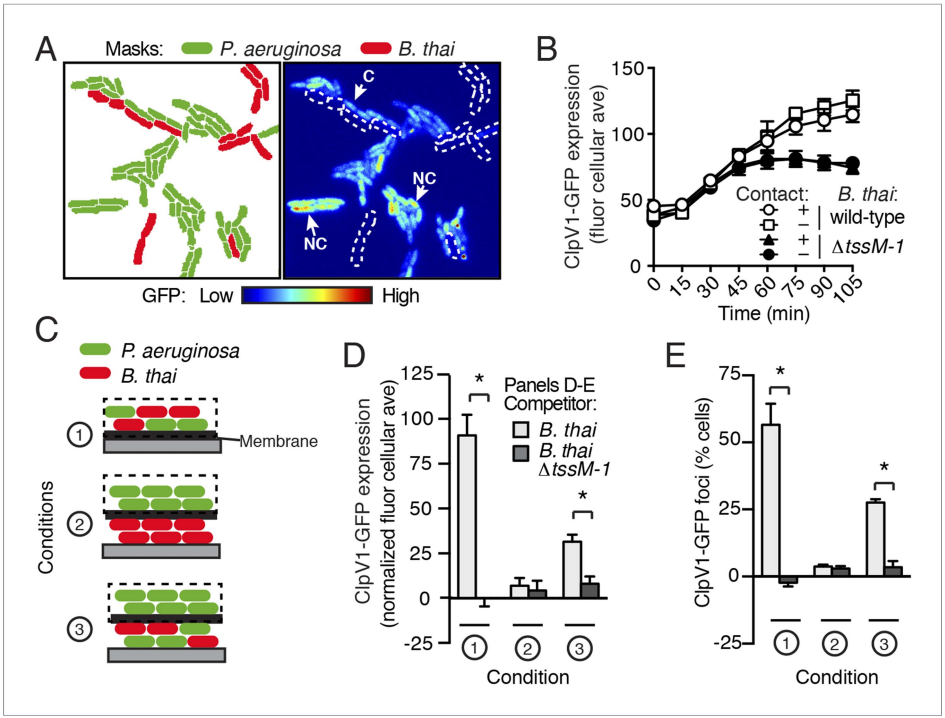

Figure 6. PARA is induced by a diffusible signal. (**A**) The PARA-associated increase in H1-T6SS expression is not contact-dependent. A representative region from a *P. aeruginosa clpV1-gfp*–*B. thai* co-culture following 120 min of growth is depicted. Cells masks are colored by cell identity (left panel); GFP intensity with *B. thai* cell positions outlined in white dashed lines (right panel). Arrows indicate *P. aeruginosa* cells contacting (**C**) or not contacting (NC) *B. thai*. (**B**) Average cellular ClpV1-GFP expression for contacting and non-contacting subpopulations described in (**A**). (**C**) Schematic depicting the experimental setup for (**D**). (**D**–**E**) PARA induction requires proximity to contacting *P. aeruginosa*–*B. thai* cells. Bacterial growth was initiated as pictured in (**C**). ClpV1-GFP was measured in populations on the membrane (black dashed lines). Average cellular ClpV1-GFP expression (**D**) and percentage of cells with foci (**E**) was determined. ClpV1-GFP expression measured in co-cultures was normalized by subtracting *P. aeruginosa* monoculture measurements. Error bars represent ±SD; n = 3 fields. Asterisks indicate significant differences between indicated groups ($p < 0.05$).

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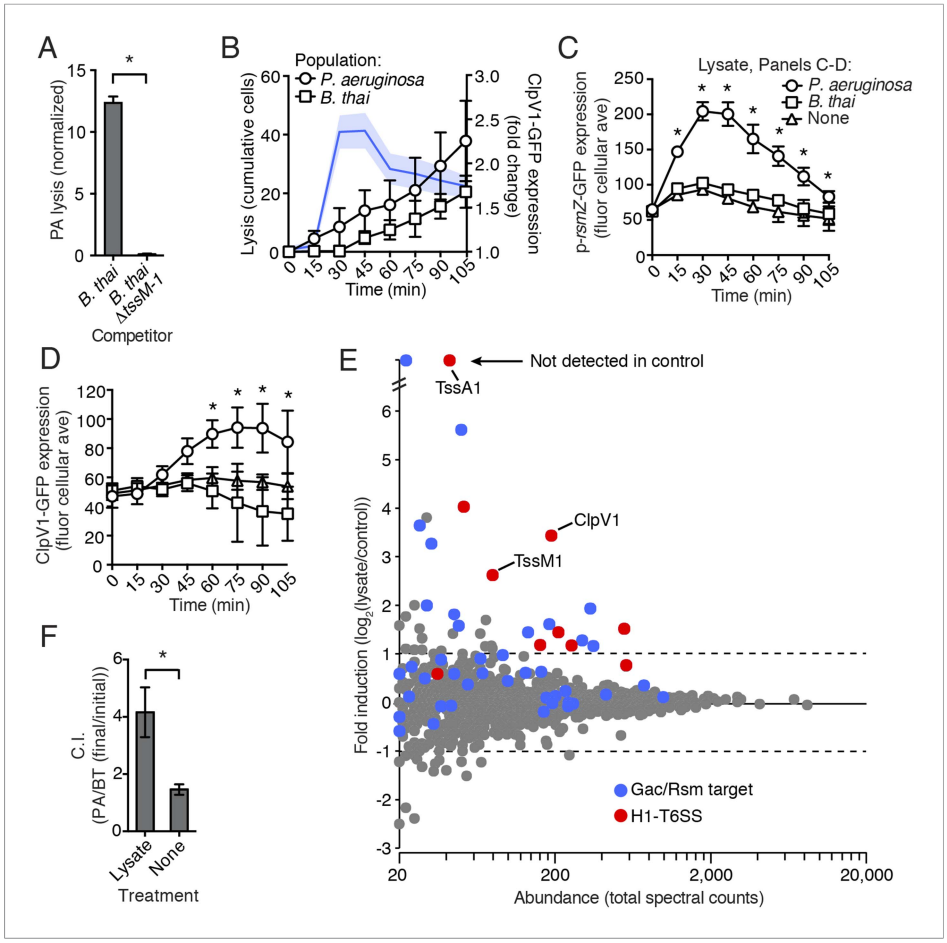

Figure 7. *P. aeruginosa* lysis is sufficient to induce PARA. (**A**) T6S^{BT} promotes *P. aeruginosa* lysis. Lysis of *P. aeruginosa* was measured under TLFM conditions and data were normalized to contacts with *B. thai*. n = 3 fields. Asterisk indicates significant difference between *B. thai* and *B. thai* Δ*tssM-1* ($p < 0.05$). (**B**) *P. aeruginosa* lysis precedes induction of H1-T6SS expression and *B. thai* lysis. Lysis (left axis) and fold increase in ClpV1-GFP (blue line, right axis) measured concurrently under TLFM conditions. ClpV1-GFP levels from *P. aeruginosa*–*B. thai* co-culture were normalized to *P. aeruginosa* monoculture. Error bars and light blue shading, ± SD. n = 4 fields. See also ***Video 6***. (**C**) *P. aeruginosa* lysate stimulates the Gac/Rsm pathway. Average cellular p-*rsmZ*-GFP expression in *P. aeruginosa* cultivated on lysate-infused growth pads. Cellular GFP expression was calculated as described in ***Figure 2***. (**D**) H1-T6SS expression is stimulated by *P. aeruginosa* lysate. Average cellular ClpV1-GFP expression of *P. aeruginosa* cultivated on lysate-infused growth pads. Corresponding H1-T6SS activity is shown in Figure 7—figure supplement 1. (**C**–**D**) n = 3 fields; asterisks indicated significant differences between *P. aeruginosa* lysate and no lysate ($p < 0.05$). (**E**) Expression of Gac/Rsm-regulated proteins is increased in lysate-treated *P. aeruginosa* cells. Quantitative mass spectrometry was used to compare the proteome of PBS (control) and lysate treated *P. aeruginosa*. Previously identified Gac/Rsm targets are indicated and H1-T6SS proteins discussed in this study are labeled. Data derive from two biological replicates. (**F**) Lysate stimulates H1-T6SS-mediated killing of *B. thai* Δ*tssM-1*. Outcome of interspecies growth competition experiments between the indicated *P. aeruginosa* and *B. thai* in the presence or absence of *P. aeruginosa*-derived lysate. Error bars represent ±SD; n = 3 co-cultures. Asterisk indicates significant difference between lysate and no lysate treatments ($p < 0.05$).

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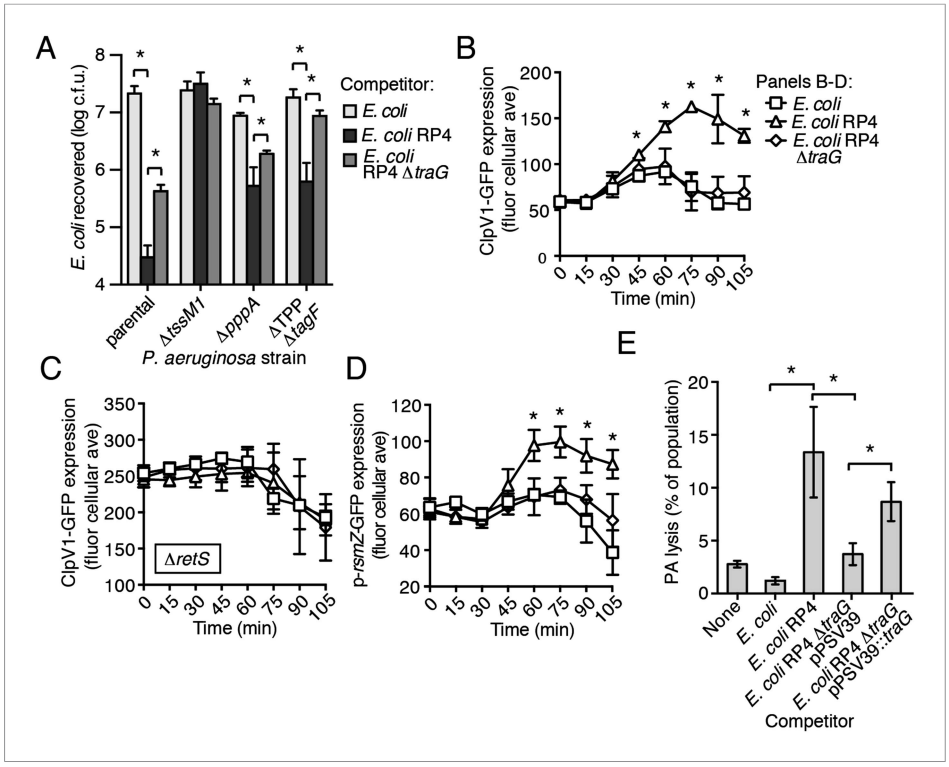

Figure 8. The RP4-encoded IncP-type T4SS induces PARA through lysis of *P. aeruginosa*. (**A**) The TPP is not required to differentially target *E. coli* with a T4SS. Outcome of growth competition experiments demonstrating increased susceptibility of T4S^{+} *E. coli* to the H1-T6SS of *P. aeruginosa*. See also ***Figure 8—figure supplement 1*** for genetic complementation data of the *traG* deletion. n = 4 co-cultures. (**B**) H1-T6SS expression is elevated in the presence of *E. coli* containing a T4SS. Average cellular ClpV1-GFP expression of the *P. aeruginosa clpV1-gfp* throughout co-culture with the indicated *E. coli strains*. (**C**) RetS is required for increased H1-T6SS expression. Average cellular ClpV1-GFP expression of the *P. aeruginosa clpV1-gfp* Δ*retS* throughout co-culture with the indicated *E. coli strains*. (**D**) *E. coli* bearing a T4SS stimulates *rsmZ* expression. Average cellular GFP levels of *P. aeruginosa* p-*rsmZ-gfp* in TLFM co-culture experiments with the indicated *E. coli* strains. (**B**–**D**) n = 3 fields; asterisks indicate significant differences between *E. coli* and *E. coli* RP4 co-cultures ($p < 0.05$). (**E**) The T4SS encoded on RP4 promotes *P. aeruginosa* lysis. Relative *P. aeruginosa attB::lacZ* lysis was measured following co-cultivation with the indicated *E. coli* strain by comparing extracellular to total β-galactosidase activity. Error bars represent ±SD; (**A**) and (**E**) n = 3 co-cultures; asterisks indicate significant differences between indicated groups ($p < 0.05$).

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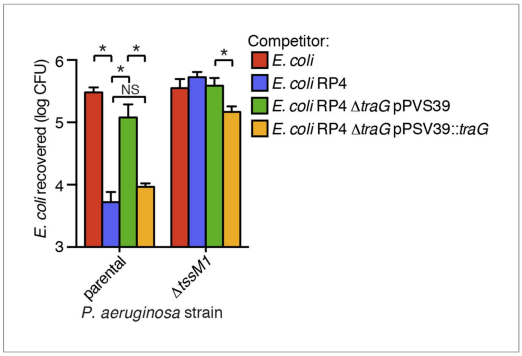

Figure 8—figure supplement 1. Genetic complementation of *traG* restores H1-T6SS-dependent targeting of *E. coli* RP4. Outcome of growth competition experiment in which *P. aeruginosa* and *E. coli* were co-cultivated under T6SS-promoting conditions. n = 4 co-cultures. Asterisks indicates significant differences between groups ($p < 0.05$); NS, not significant.

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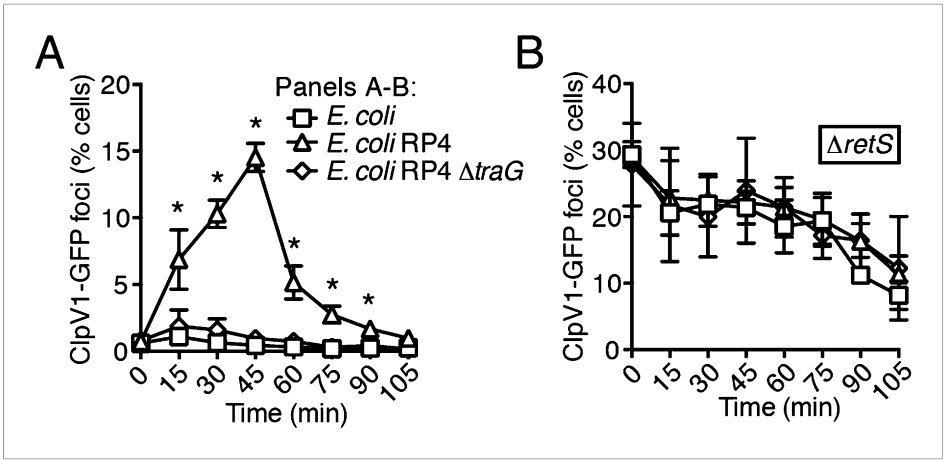

Figure 8—figure supplement 2. The RP4-encoded T4SS induces a PARA-associated increase in H1-T6SS activity. Percentage of cells containing ClpV1-GFP foci by a *P. aeruginosa clpV1-gfp* strain cultivated with the indicated competitor. Error bars represent ±SD; n = 3 co-cultures. Asterisks indicate significant differences between co-cultures with *E. coli* and *E. coli* RP4 ($p < 0.05$).

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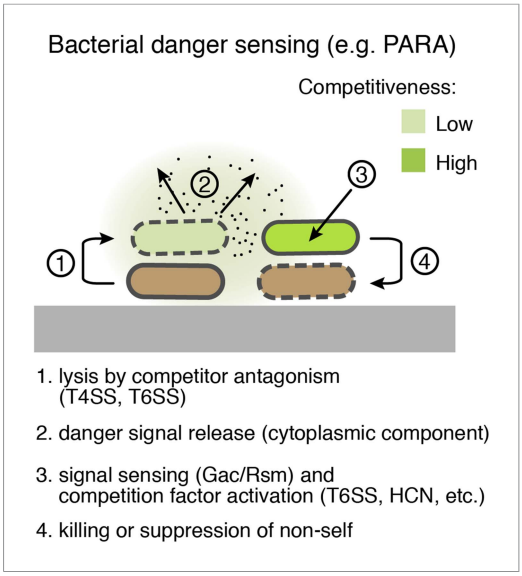

Figure 9. Bacterial danger sensing. The model depicts antagonism between two species of bacteria, represented in green and brown. The green cells possess a danger sensing pathway; specifics of PARA are provided in parentheses.

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