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SUPPORTING INFORMATION (SI)

Strain construction. Fluorescent fusions to endogenous genes were constructed as follows: The 500 bp regions flanking the C-terminus (stop codon) of each target gene was amplified. These flanking regions were spliced together using a BamHI site (GGTACC) replacing the stop codon of the target gene. (The BamHI sequence acted as a two residue linker between the protein and fluorescent protein label.) The spliced fragments were then cloned into the vector pEXG2 (1). Either mCherry or superfolder-GFP (sfGFP) was cloned into the BamHI site to construct a C-terminal fluorescent fusion to the target genes *clpV*, *tssB*, and *fha*. Finally, each fused gene was inserted at the endogenous promoters by allelic exchange, as described in Ref. (1).

An in-frame deletion of *retS* was introduced to the ClpV-mCherry/TssB-sfGFP strain using allelic exchange as described in (2).

Bacterial cultivation for strain construction was performed in Luria broth (LB) medium supplemented with 25 μg/ml irgasan, 30 μg/ml gentamycin, and counter selection for allelic exchange was performed on low-salt LB supplemented with 5% wt/vol sucrose.

Behavior of ClpV-TssB double fusion. The ClpV-mCherry/TssB-GFP double fusion measurably changed ClpV behavior compared to the ClpV-mCherry fusion alone. Although the single-label ClpV-mCherry focus lifetime is identical in both wild type and Δ*retS*, the ClpV-mCherry/TssB-GFP foci lifetimes are 10% longer in Δ*retS* where T6SS is over-expressed. Additionally, in wild type, the ClpV-mCherry/TssB-GFP strain has a diminished firing rate compared to the ClpV-mCherry fusion alone, with at most one ClpV spike seen over the 7–9 minute experiment with a spike lifetime five times as long as wild type ClpV-mCherry. Although this observation of less active dynamics might suggest loss-of-function, we performed a competition between *B. thailandensis* and the double-labeled wild type and the double-labeled and wild type cells had statistically indistinguishable fitness. We therefore believe that the qualitative localization dynamics observed in ClpV-mCherry/TssB-GFP Δ*retS* is informative, despite the failure of TssB-GFP to completely complement TssB with respect to the ClpV dynamics.

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