

6S RNA facilitates bacterial virulence and adaptation at the epithelial barrier

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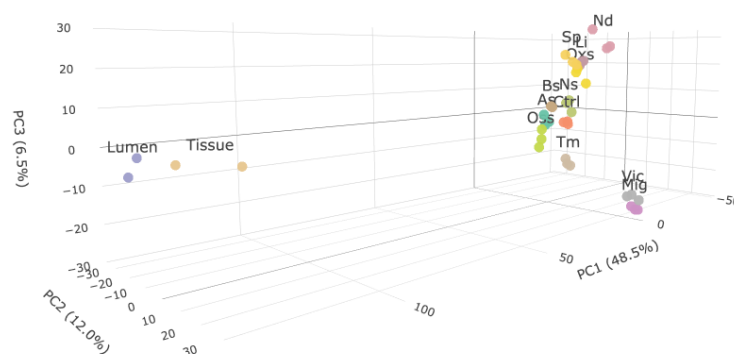
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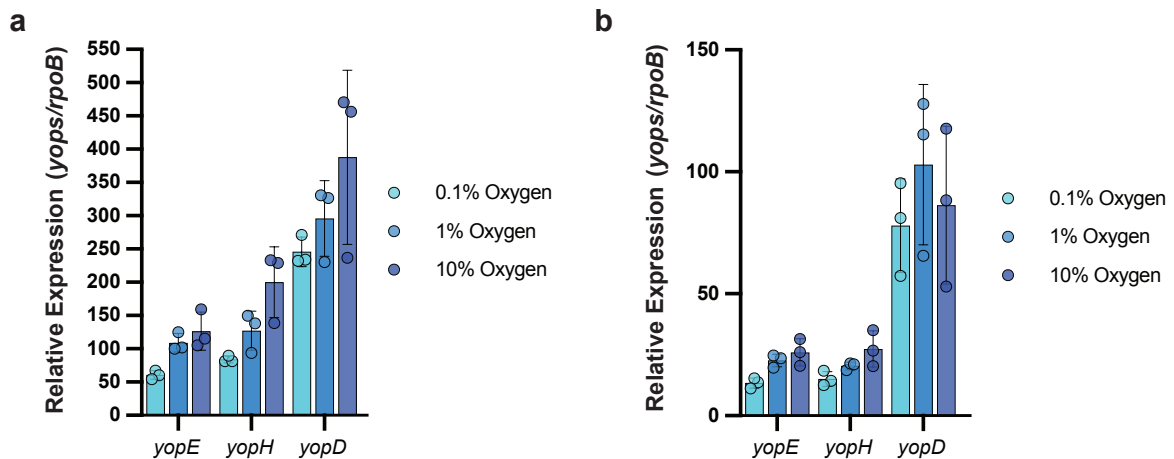
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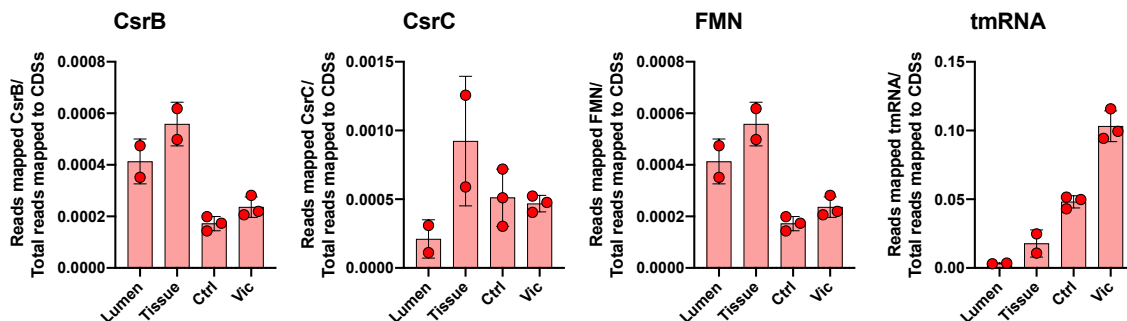
Supplementary Information



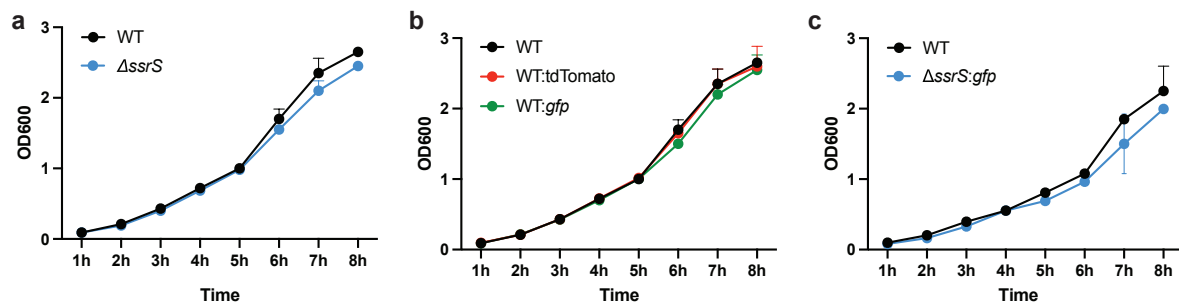
Supplementary Figure S1. Principal component analysis (PCA) of *Yersinia pseudotuberculosis* transcriptomes under infection-relevant stress conditions and *in vivo* environments with distant clustering of *in vivo* transcriptomes. PCA was performed using TPM values from the PATHOgenex dataset (Avican et al., 2021), which includes RNA-seq data from bacteria exposed to various *in vitro* stressors along with control condition. Each *in vitro* condition includes three biological replicates. Additionally, transcriptomes from bacteria isolated from cecal lumen and tissue of two infected mice are shown. Different stressors are acidic stress (As), bile stress (Bs), control (Ctrl), low iron (Li), microaerophilic growth/hypoxia (Mig), nutritional downshift (Nd), nitrosative stress (Ns), osmotic stress (Oss), oxidative stress (Oxs), stationary phase (Sp), temperature (Tm), and virulence-inducing condition (Vic).



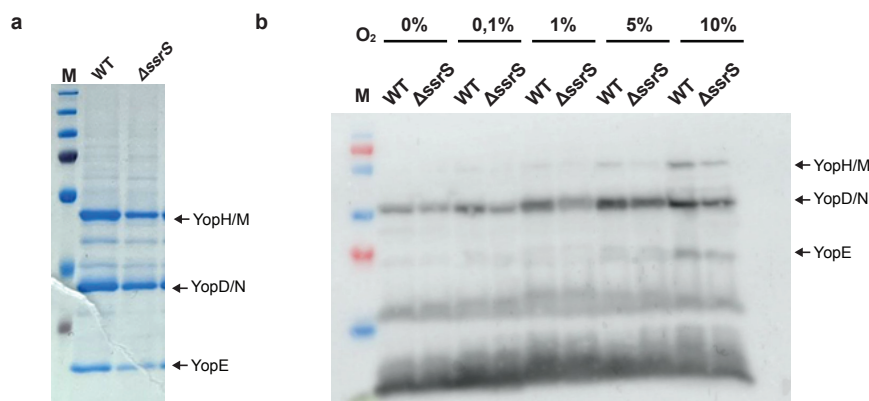
Supplementary Figure S2. The expression of T3SS genes increases upon increased oxygen concentration. **a-b** Relative expression of *yopE*, *yopH*, and *yopD* under T3SS inducing (37°C, Ca²⁺ depletion) **(a)** and non-inducing (37°C) **(b)** condition in 0.1%, 1%, and 10% oxygen concentrations for 3 hours measured with qPCR. Relative expression of *yopE*, *yopH*, and *yopD* are calculated according to Ct values normalized to that of *rpoB*. All qPCR experiments are performed with three biological and three technical replicates. Data are presented as mean values \pm SD from 3 sets (n=3) for each condition.



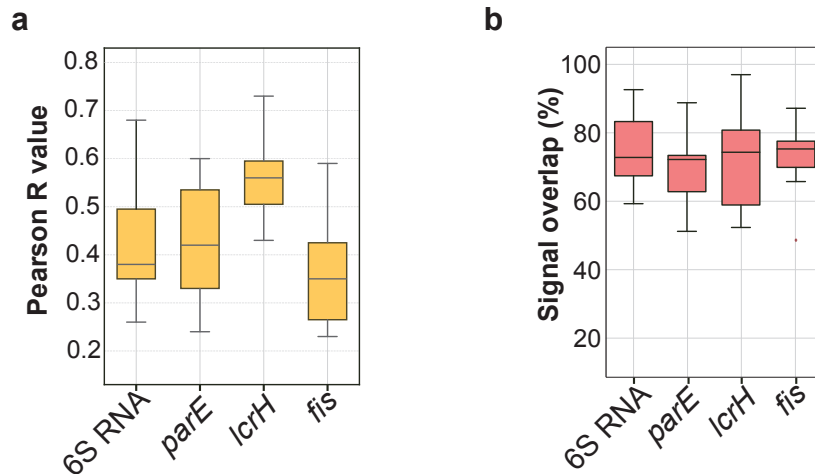
Supplementary Figure S3. Expression levels of CsrB, CsrC, FMN, and tmRNA in lumen and tissue located bacteria and also in *in vitro* environmental (26°C) and virulence (T3SS) inducing condition from PATHOgenex dataset (Avican et al., 2021). Gene expression levels were calculated as the ratio of total number of reads mapped each non-coding RNA to total number of reads to all CDs in *Y. pseudotuberculosis*. The data are presented as mean values \pm SD from 2 sets of specimens (n=2) for lumen and tissue samples and 3 biological replicates for *in vitro* conditions (n=3).



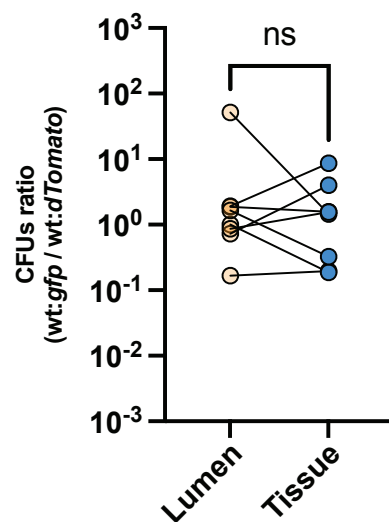
Supplementary Figure S4. Growth curves of *Y. pseudotuberculosis* wild-type and mutant strains used in this study. All strains exhibited comparable growth dynamics, reaching stationary phase with no or negligible differences (slight growth defect for *ssrS* mutant) in growth rates, indicating that the introduced mutations and reporter constructs did not affect general fitness under these conditions. Cultures were initiated with 1:100 dilutions of overnight cultures and grown in LB medium at 26 °C with aeration for 8 hours. Optical density at 600 nm (OD₆₀₀) was measured hourly to monitor bacterial growth. **a** WT and *ssrS* deletion mutant. **b** WT, WT:*tdTomato*, and WT:*gfp*. **c** WT and *ssrS:gfp*. Three biological replicates (n=3) were used in **a**, **b**, and **c**.



Supplementary Figure S5. T3SS expression and secretion in *Y. pseudotuberculosis* WT and *ΔssrS* mutant strains under T3SS-inducing condition at varying oxygen levels. **a** SDS-PAGE analysis of secreted proteins (secretome) from WT and *ΔssrS* strains under T3SS-inducing conditions (37 °C, Ca²⁺ depletion) at atmospheric oxygen levels (21%) for 3 hours. **b** Western blot detection of T3SS effector proteins levels by WT and *ΔssrS* strains' pellets under T3SS-inducing condition (37 °C, Ca²⁺ depletion) across a range of low oxygen concentrations (0%, 0.1%, 1%, and 5%) for 3 hours.



Supplementary Figure S6. Computational analysis of RNAscope assay reveals heterogeneous expression of 6S RNA, *parE*, *lcrH*, and *fis* in *Y. pseudotuberculosis*. **a** Pearson correlation analysis between green fluorescence signals from RNAscope probes targeting *Y. pseudotuberculosis* 23S rRNA and red signals from probes targeting 6S RNA, *parE*, *lcrH*, and *fis*. **b** Quantification of the percentage of between red signals (6S RNA, *parE*, *lcrH*, and *fis*) that spatially overlap with green 23S rRNA signals, indicating the degree of transcript co-localization within bacterial cells.



Supplementary Figure S7. Co-infection of mice with WT *Y. pseudotuberculosis* strains ectopically expressing fluorescent reporter proteins do not have an effect on infection outcome. Ratio of CFUs recovered from cecal tissue and lumen compartments of mice (n=8) co-infected with 1:1 mixture of *Y. pseudotuberculosis* WT:gfp and *Y. pseudotuberculosis* WT:tdTomato strains. The two strains were distinguished with fluorescent signal by GFP

expression and by tdTomato expression in WT strain. Statistical significance between two groups was calculated via Mann-Whitney test.