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Title: Robustness of a model microbial community emerges from population structure among single cells of a clonal population.

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Running title: Single cell gene expression

Originality-Significance Statement

This work is original in its combination of microbial physiology experiments with cutting edge single cell gene expression analysis in an environmentally relevant model microbial community. We found that the loss of population structure among single cells in one member of a model microbial community can lead to the collapse of the entire community. Our conclusions may be relevant to understanding how more complex microbial communities respond to change in their environments, which is critical towards fundamental understanding of microbial communities and the successful use of their ecosystem functions in industrial, medical, and environmental applications.

Keywords: single cell, robustness, gene expression, *Desulfovibrio*, syntrophy, sulfate respiration.

Summary

Microbial populations can withstand, overcome, and persist in the face of environmental fluctuation. Previously we demonstrated how conditional gene regulation in a fluctuating environment drives dilution of condition-specific transcripts, causing a population of *Desulfovibrio vulgaris* Hildenborough (DvH) to collapse after repeatedly transitioning from sulfate respiration to syntrophic conditions with the methanogen *Methanococcus maripaludis*. Failure of the DvH to successfully transition contributed to the collapse of this model community. We investigated the mechanistic basis for loss of robustness by examining whether conditional gene regulation altered heterogeneity in gene expression across individual DvH cells. We discovered that robustness of a microbial population across environmental transitions was attributable to the retention of cells in two states that exhibited different condition-specific gene expression patterns. In our experiments, a population with disrupted conditional regulation successfully alternated between cell states. Meanwhile, a population with intact conditional regulation successfully switched between cell states initially, but collapsed after repeated transitions, possibly due to the high energy requirements of regulation. These results demonstrate that the survival of this entire model microbial community is dependent on the regulatory system's influence on the distribution of distinct cell states among individual cells within a clonal population.

Introduction

In order to understand microbial functions within ecosystems, we must understand the mechanisms by which microorganisms interact with one another as a community, respond to perturbations, and signal approaching collapse or tipping points (Dai et al. 2012). A central property in this regard is the ability of microbial communities to maintain function in face of dynamic fluctuations in nutrient and energy resources (Kitano 2004; Fuhrman et al. 2015; Song et al. 2015). Such fluctuations of resource availability in space and time are fundamental features of numerous environments, including many ecologically and economically relevant microbial systems (McClain et al. 2003). Understanding how fluctuations in resource availability affect microbial community structure and growth is very important towards understanding microbial systems.

Conditional gene regulation, or the control of gene expression in response to specific conditions, has been hypothesized to be important for adaptation and response of microbial communities to environmental perturbations (Futuyma & Moreno 1988). There is building evidence from several studies that regulatory elements accumulate mutations during the evolution of improved growth under stable resource regimes (Hindré et al. 2012; Blount et al. 2008; Cooper & Lenski 2000; Hillesland & Stahl 2010; Lee et al. 2009; Hottes et al. 2013; Kurlandzka et al. 1991; Yang et al. 2011; Hillesland et al. 2014). For example, the sulfate reducing bacterium *Desulfovibrio vulgaris* Hildenborough (DvH) was grown for 1000 generations in syntrophic co-culture with the methanogen *Methanococcus maripaludis (Mmp)* without sulfate available as an electron acceptor. In the absence of sulfate, DvH is dependent on the consumption of its fermentation products by Mmp to obtain energy for growth on lactate (Bryant et al. 1977). This type of syntrophic

relationship is also prevalent in nature (Plugge et al. 2011; Steger et al. 2011; Raskin et al. 1996). Under these stable conditions, DvH gained mutations in many sulfate respiration pathway genes (Hillesland et al. 2014), in particular mutations were enriched in the coding regions of regulatory genes (Turkarslan et al. 2017).

In light of these observations, in a previous study we investigated whether conditional regulation contributes to robustness of a model microbial population growing under variable conditions in contrast to the stable conditions noted above. One of the DvH regulatory genes that accumulated mutations during evolution in syntrophy (DVU0744 - a sigma-54 family transcription factor), was identified as a potential novel transcriptional regulator of sulfate respiration using a network model of gene expression under a range of conditions and a DVU0744 transposon insertion mutant was generated (Turkarslan et al. 2017). The growth rate of the DVU0744::Tn5 mutant was reduced relative to wild type under conditions of excess sulfate. To test the importance of conditional regulation on the survival of a model microbial community in variable conditions, we established co-cultures of DvH wild type and DvH DVU0744::Tn5 with *Methanococcus maripaludis*, and investigated the robustness of these two-organism model microbial communities during growth in repetitively fluctuating conditions that either supported independent growth of DvH via sulfate respiration (SR) or required interdependent growth of the two organisms in syntrophy (ST). We found that all replicates of the wild type DvH co-culture were unable to persist with repeated transfer between these growth states, some collapsing after as few as 3 SR/ST transitions. Remarkably, all replicates of a co-culture with DvH DVU0744::Tn5 persisted across the same transitions without collapse (Turkarslan et al. 2017). A series of

additional measurements were used to explore the mechanism underlying this collapse in wild type and persistence in the mutant. A model was developed that predicted decrease in cellular concentrations of essential proteins in wild type cells as they transitioned between ST and SR conditions, due to active conditional regulation. In contrast, mutant cells with disrupted conditional regulation (DVU0744::Tn5), demonstrated "leaky" expression of genes that are normally repressed by DVU0744 in ST conditions, allowing carryover of SR essential proteins from ST conditions to jumpstart growth. Global proteomics and initial analysis of single cell gene expression reflected model predictions, displaying decreased, or "diluted", protein and transcript abundance in wild type cells after repeated transitions. In addition, the amount of heat production measured through microcalorimetry was much higher for wild type than the mutant, suggesting that conditional regulation imposed a greater energetic burden on cells during a fluctuating resource environment, leading to depletion in abundance of condition-specific transcripts and proteins and collapse (Turkarslan et al. 2017).

The purpose of the present study was to further investigate whether collapse of the wild type DvH population during repetitive resource fluctuations was a consequence of altered population structure with regard to heterogeneity in gene expression between single cells, beyond simply diluted transcript abundance noted in Turkarslan et al. (2017). Technological advances have made it possible to examine gene expression in microorganisms at the single cell level and have uncovered stochastic processes, heterogeneity among single cells, and relationships between mRNA and protein abundance (Blake et al. 2006; Taniguchi et al. 2010; Cai et al. 2006). This body of work has revealed

the importance of heterogeneity at the single cell level in the emergence of population level properties such as growth rates, yield, resilience, and robustness during dynamic growth conditions (Buettner et al. 2015; Delvigne & Goffin 2014; Paszek et al. 2010; Kellogg & Tay 2015).

We sought to investigate the impact of disrupted conditional gene regulation on the heterogeneity of gene expression among single cells of a clonal population during fluctuating resource conditions. We examine robustness as a general concept that incorporates properties such as resistance and resilience (Song et al. 2015), adhering to the definition of robustness as "a property that allows a system to maintain its functions against internal and external perturbations" (Kitano 2004). Gene expression patterns in single cells were measured using a microfluidic device and those patterns then used to identify specific "states" of individual DvH cells and proportions of cells in each state within the community. Our results offer insight into patterns of heterogeneity among single cells in the context of conditional regulation and robustness in a model syntrophic association. This model system is representative of microbial communities in many anaerobic environments such as ruminant digestive systems, anaerobic digesters, and sediments (Stolyar et al. 2007; Schink 1997). Thus, these analyses provide basic understanding of mechanisms contributing to the robustness of microbial communities.

Results

Growth across syntrophic (ST) and sulfate respiration (SR) conditions. Growth of *Desulfovibrio vulgaris* (DvH) and *Methanococcus maripaludis* (Mmp) was measured in the

mutant and wild type co-cultures by OD as they were transferred alternately between syntrophic (no sulfate) and sulfate respiration (sulfate available) conditions. All three replicates of the mutant strain maintained consistent growth rates across all 7 ST/SR transitions while one wild type replicate collapsed (no detectable growth) at ST transfer 4 (ST4), after 3 ST/SR transitions (Figure 1). All three wild type replicates collapsed by ST6. Across all transfers, we observed faster growth in SR conditions than in ST conditions in strains. At the first transfer from ST to SR (ST1/SR1), all wild type replicates grew faster than the mutant. The wild type replicate that collapsed earliest at ST4 (replicate "2") was chosen for single cell analysis alongside the corresponding mutant replicate at the first transition (ST1 to SR1) and at the third transition (ST3 to SR3), prior to collapse of the wild type in ST4 (Figure 1). The relative abundance of Mmp and DvH remained constant in all growing cultures with Mmp at approximately $1/4^{th}$ the concentration of DvH, measured by microscopic cell counts, as published in Turkarslan et al (2017). These growth data are consistent with similar experiments testing wild type co-cultures against a set of conditional regulatory mutants for RNA-Seq analysis (Turkarslan et al. 2017).

Single cell rRNA gene expression screening and analysis. For all time points selected for single cell analysis (Figure 1), a subset of the 80 sorted cells were selected for further analysis if they expressed both 16S and 23S rRNA genes, totaling 488 single cells across all populations (Supplemental Figure 12). It is unknown whether absence of 16S and/or 23S rRNA expression in some single cells was due to low activity or a technical issue, such as failed sorting, cell lysis, or cDNA synthesis. The number of cells in which expression of both rRNA genes was detected was higher in the mutant populations (range 65-69 cells, mean

66.5 cells) than wild type populations (range 51-59 cells, mean 55.5 cells) (p-value < 0.01, Student's t-test). For each cell, 16S and 23S rRNA gene expression was positively correlated (Supplemental Figure 12). Cells exhibited heterogeneity in the number of rRNA transcripts expressed per cell, ranging from 2-557 16S rRNA transcripts per cell and 7-1,800 23S rRNA transcripts per cell (Figure 2). Population expression distributions varied from unimodal to multimodal patterns (Figure 2). In the ST1/SR1 transition, mean expression of 16S and 23S rRNA genes in single cells increased in both wild type and mutant (Figure 2). However, in the ST3/SR3 transition mutant cells increased expression of both rRNA genes similar to the ST1/SR1 transition, while in the wild type ST3/SR3 transition 23S rRNA expression barely increased and 16S rRNA expression decreased, as expected from growth rates (Figure 1).

Relative quantity (RQ) of transcripts per cell across all conditions. For cells expressing both 16S and 23S rRNA genes, mRNA transcript levels per cell were very low. Of the transcripts expressed above the limit of detection (1 molecule per cell, see Experimental Procedures), each cell contained an average of 1.97 mRNA molecules (RQ) from a given gene ($\log_2$RQ=1 for a cell expressing 2 mRNA molecules of a particular gene) (Figure 3). If we include transcripts that were not expressed above the limit of detection, the average expression level of each transcript per gene per cell was 0.4 molecules (data not shown).

Frequency of single cells expressing different numbers of genes. The number of different genes that were expressed at any RQ was determined for each cell (Figure 4). The number of expressed genes per cell ranged from 2 (16S and 23S rRNA genes only) to 80 out of the

88 total genes we assayed. Average counts of the number of expressed genes per cell varied across each population (Figure 4). The frequency of cells (y-axis) with more expressed genes increased in both mutant and wild type in the ST1/SR1 transition (Figure 4A-B), though wild type expressed more genes per cell than the mutant (Figure 4C). This result is consistent with the higher growth rate of wild type in SR1 (Figure 1) and the increased rRNA gene expression in wild type during the ST1/SR1 transition (Figure 2). In the ST3/SR3 transition the mutant behaved similarly to the ST1/SR1 transition with an increase in the number of expressed genes per cell in SR3 (Figure 4E). In contrast, in the wild type ST3/SR3 transition, the number of expressed genes per cell decreased in SR3 (Figure 4D). Furthermore, the SR3 mutant population, which continued growth in subsequent transfers, expressed more genes per cell than WT in the SR3 conditions (Figure 4F), a reversal of the result observed in SR1 (Figure 4C). This result is consistent with the greater rRNA gene expression in mutant than wild type in SR3 (Figure 2). This molecular signature of collapse was not recapitulated at the phenotype level as both mutant and wild type had similar growth rates and OD at transfer (Figure 1C).

Defining states of single cells based on gene expression profiles. Principal component analysis (PCA) was used to visualize differences between single cells from each population and growth condition (Figure 5). We found that in each population, single cells were heterogeneous in their expression patterns. We observed regions of the PCA plots that exclusively contained cells from the ST1 condition (called “state A”), cells from the SR1 condition (called “state B”), or contained both ST and SR cells (called “state C”) (Figure 5A). The “state space” for each cell state was defined for wild type when the cultures were

growing well in ST1 and SR1 (Figure 5A). Then, the boundaries of the "state space" was superimposed onto the PCA plots for other single cells in populations including mutant ST1/SR1 (Figure 5B), wild type ST3/SR3 (Figure 5C), and mutant ST3/SR3 (Figure 5D).

We observed striking changes in the number of cells in each state across the ST/SR transitions. In the mutant that grew across all transitions, the number of cells in state A and B were inversely correlated for all transitions (Figure 6). In ST conditions, state A cells increased in abundance and state B cells decreased in abundance. In SR conditions state B cells increased in abundance while state A cells decreased in abundance (Figure 6). Initially, we observed the same pattern in the wild type, with a much more dramatic shift in the relative proportions of cells in state A and B from ST1 to SR1 to SR3 than observed in the mutant (Figure 6). However, in contrast to the mutant population, in wild type SR3, prior to collapse, state A cells remained elevated in abundance rather than decreasing. And, state B cell abundance increased only slightly rather than increasing markedly as in SR1. In summary, wild type single cell population structure shifted dramatically between state A and B initially, but when approaching collapse there was little change in the relative abundance of the cells in each state. Meanwhile, the mutant population, though it exhibited less dramatic shifts between cell states A and B initially, continued to alternate between the two cell states with environmental shifts for the duration of our experiment.

Gene expression of cells from each cell state. We wondered what genes, or gene expression patterns, defined the different cell states we observed (states A, B, and C) and questioned how the loss of the state B cells, and lingering of state A cells, contributed to population

collapse in wild type after SR3. We found two genes DVU0847 (adenylyl-sulfate reductase, alpha subunit) and DVU2405 (iron-containing alcohol dehydrogenase) that were significantly differentially expressed (p-value < 0.05, Student's t-test) between state A and state B cells. In addition, state A cells were up-regulated relative to state C cells in DVU2405 and DVU0847 as well as DVU1295 (sulfate adenylyltransferase, *sat*) and DVU2399 (putative hydrogenase) (Figure 7B). State B cells expressed higher levels of *sat* relative to cell state C cells. State A and B cells had in common higher expression of *sat* relative to state C cells. We also compared the number of different genes expressed by cells from each state in wild type ST1/SR1. Interestingly, we found that whereas state A cells expressed few genes at high levels, significantly greater number of condition-specific genes were expressed in state B cells (Figure 8).

Discussion

Microbial populations experience constant perturbations that affect their relationship to the environment, intrinsic state, and ecosystem function (Song et al. 2015). The nature of response to perturbations is a fundamental property of microbial communities. Using a model microbial community, we aimed to determine how conditional regulation influences the robustness of a simple community by analyzing gene expression among single cells during repeated environmental transitions.

We found that the robustness of a two-organism model microbial community across repeated transitions relies on small subpopulations of cells in distinct states expressing condition-specific genes (Figure 5-8). Meanwhile, a larger population of cells remains the

same across conditions without sharing a distinct pattern of gene expression. In our experiments, wild type DvH exhibited dramatic and robust switching between two cell states early in the experiment (Figure 6, ST1 and SR1), while growing robustly (Figure 1) and elevating rRNA gene expression for translation (Figure 2). However, after repeated transitions, wild type cells failed to alternate between states to match the altered growth conditions. When the numbers of cells in one condition-specific cell state (state B – those expressing many condition-specific genes under sulfate respiration conditions) dropped below a critical frequency the population was unable to grow in the next transition and collapsed. In contrast, a regulatory mutant exhibited less dramatic shifts between the abundance of cells in each of the states (Figure 6), and overall slower growth relative to wild type (Figure 1), but was able respond to new conditions with expression of condition-specific genes by a small set of cells and maintain growth across transitions.

Our results offer a novel single cell perspective into the effect of conditional regulation on robustness, which is relevant to discovering mechanisms that control survival of microbial communities across resource fluctuations. For an infrequent shift in conditions, the extra energy and nutrient resources required to meet that challenge by producing condition-specific cells is advantageous, resulting in increased growth rates and/or yield. These data raise the question of the consequences of differences between the frequency of fluctuations and the rate at which conditional regulation can act. If rate of regulation and the rate of fluctuation are disparate, cells may not be able to respond to resource shifts appropriately, leading to population collapse. Meanwhile, a mutant population — with an altered regulatory system— produces a smaller proportion of condition-specific cells in response

278 to new conditions, and will persist through repeated fluctuations. Our previous work
279 showed lower heat output by the mutant relative to wild type, suggesting that the
280 production of large proportions of cells with condition-specific gene expression patterns
281 (whether many expressed genes in state B, or a few highly expressed genes in state A)
282 imposes a significant energetic burden (Turkarslan et al. *2017)*.
283
284 If resource fluctuations are slower than the system's intrinsic dynamics, microbial
285 communities may be capable of adjusting appropriately to the new environment and still
286 maintain function (Song et al. 2015). This is likely the case in many natural microbial
287 assemblages that inhabit variable resource environments (Fuhrman et al. 2015), and may
288 be pertinent to predict how microbial communities will respond to future perturbations
289 and understand how they have responded to past perturbations. Thus, future experiments
290 examining different rates of environmental transitions and different energy resources will
291 contribute to fundamental understanding of tipping points, where the rate of fluctuation
292 exceeds the intrinsic capabilities of the cells' regulatory system and energy stores. The
293 effect of genetic diversity on microbial community response to fluctuating resource
294 availability must also be considered to place the results of this work in the context of
295 natural assemblages. As most microbial communities are highly diverse, response to
296 fluctuating resources could occur simultaneously on multiple scales as distinct cells,
297 species, or even sub-communities distribute themselves over time into distinct
298 physiological states driven by the environment in contrast to the two-organism system
299 studied here. Furthermore, community structure of microbial communities can shift
300 rapidly in response to small environmental changes (Ward et al. 2017), adding an

301 additional challenging dimension to predicting the role of single cell heterogeneity and
302 conditional regulation in persistence of microbial communities through environmental
303 fluctuations.
304
305 These results are also relevant towards understanding how the Allee effect, defined as
306 negative density-dependent growth below a population threshold (Allee et al. 1949; P. A.
307 Stephens W. J. Sutherland 1999), works in microbial populations. For the DvH/Mmp
308 system, our data suggests that the structure of the cell population with regard to single cell
309 gene expression patterns is important for growth after dilution or bottlenecking. Therefore,
310 not only the total density of cells, but also the density of heterogeneous groups of
311 individual cells, controls growth of the microbial community during subsequent
312 environmental fluctuations.
313
314 It remains unknown what mechanism cells use to reach distinct cell states. There is strong
315 evidence for the role of stochasticity in gene expression at the single cell level (Elowitz et
316 al. 2002; Swain et al. 2002; McAdams & Arkin 1997; Spudich & Koshland 1976). Therefore,
317 it is possible that stochastic processes are behind at least some of the heterogeneity in gene
318 expression we observe. Also, the low numbers of mRNA transcripts per cell we detected
319 are consistent with other studies that quantified mRNA transcripts per cell in other
320 prokaryotic microorganisms such as *E. coli* (Taniguchi et al. 2010), *Burkholderia*
321 *thailandensis* (Kang et al. 2011), and DvH (Qi et al. 2014). While we did not measure
322 protein expression at the single cell level, it is also known that many important proteins are
323 expressed at low levels and are controlled by stochastic processes (Ghaemmaghami et al.

2003; Guptasarma 1995; Cai et al. 2006). It remains unknown whether the gene expression of the single cells we measured was influenced by any mutations at the single cell level that would affect gene expression levels. Overall, our work suggests that stochastic processes at the single cell level could affect population-level characteristics such as growth, energy use, and robustness across fluctuating conditions.

In conclusion, we found that robust growth of a model microbial community across fluctuating resources was supported by the emergence of condition-specific cell states in one organism (DvH) as defined by patterns of gene expression in a set of 88 condition-specific genes. These findings with *Methanococcus* and *Desulfovibrio* offer novel insight into how heterogeneity in gene expression among single cells supports robustness in a simple microbial community and highlights the tradeoffs associated with conditional regulation in a fluctuating resource environment. Future work will examine if these properties of a two-member microbial community expand to more diverse microbial communities where several genotypes (or species) can fulfill the same metabolic functions independently or through interactions with another organism.

Experimental Procedures

Growth medium and assessment. Cultures of DvH and Mmp were obtained and grown as previously described (Turkarslan et al. 2017). Briefly, all cultures were grown at 37 °C on CCMA medium (Walker et al. 2009) with the following modifications. Medium for syntrophic (ST) growth contained 40 mM of lactate without sulfate. Medium for sulfate respiration (SR) contained 40 mM lactate and 15 mM sulfate. Headspace consisted of 80%

347 N_2 and 20% CO_2 to create an anoxic environment and pH was adjusted to 7.2 with
348 bicarbonate. Cell concentration of both partners DvH and Mmp was monitored by OD and
349 flow cytometry. For transitions between ST and SR, co-cultures were initially grown in ST
350 medium until early log phase (OD ~ 0.15) then 0.5 mL of inoculum was transferred into 10
351 mL of fresh SR medium. Co-cultures were grown to early log density (OD600 ~ 0.2) and 0.5
352 mL was transferred back into fresh ST medium or SR medium repeatedly (Figure 1).
353
354 *Sampling for single cell gene expression.* At each transfer point, samples were collected for
355 single cell gene expression analysis by anaerobically sampling then immediately freezing
356 0.5 mL aliquots of culture in liquid nitrogen with storage at -80 °C.
357
358 *Flow cytometric cell sorting.* Cell sorting of single DvH cells from ST and SR conditions was
359 carried out as previously described (Thompson et al. 2015). Briefly, a BD Influx high-speed
360 cell sorter equipped with a small particle detector was prepared for clean single cell
361 genomics work following previously published protocols (Rodrigue et al. 2009) then used
362 to distinguish DvH and Mmp cells based on differences in forward scatter (proxy for size)
363 and side scatter (proxy for shape or granularity) properties. Single DvH cells were sorted
364 into individual wells of 96-well plates containing lysis buffer using 1.0 Drop Pure sort
365 mode.
366
367 *Single cell quantitative RT-PCR with the Fluidigm 96.96 Dynamic Array.* Single-cell
368 transcriptional changes for 88 DvH genes (Supplemental Table 1) were tracked across
369 SR/ST transitions using protocols developed for the Fluidigm 96.96 Dynamic Array

(Fluidigm Inc., South San Francisco, CA). The Fluidigm 96.96 Dynamic Array is a microfluidic device that is capable of combining amplified cDNA template from up to 96 samples with reagents for up to 96 distinct qPCR assays in a total of up to 9,216 unique quantitative PCR reactions. Assays used in this study include 88 genes (Supplemental Table 1) identified based on previously published whole-genome expression profiles where they were identified as essential to sulfate respiration in DvH (Turkarslan et al. 2017). Essentiality was determined based on Rapid Transposon Liquid Enrichment Sequencing and an associated model for essentiality (Fels et al. 2013). Control genes, 16S and 23S rRNA genes, were also included. The final set of 88 genes (Supplemental Table 1) was a subset of 120 assays that were initially tested for amplification specificity. To check amplification specificity, assays were examined for the production of non-specific products or cross-reactivity with other primers using melting curve analysis. To prepare cDNA, cells were sorted directly into a lysis/RT buffer solution consisting of 1X VILO Reaction Mix (Life Technologies), 6U SUPERase-In (Life Technologies), 0.5% NP-40 (ThermoScientific), and nuclease-free water (TEKnova) in a 96-well plate format. Sort plates were centrifuged, vortexed for 15 seconds, then frozen on dry ice and stored at -80°C. Following cell lysis and RNA denaturation (90 seconds at 65°C) reverse transcription (RT) was carried out with 1X SuperScript Enzyme Mix (Life Technologies) and T4 Gene 32 Protein (New England BioLabs, Beverly, MA) by the following program: 25 °C for 5 minutes, 50 °C for 30 minutes, 55 °C for 25 minutes, 60 °C for 5 minutes, and 70 °C for 10 minutes. cDNA was amplified in a multiplexed specific target amplification (STA) reaction with the 88 DvH gene primer pairs using TaqMan® PreAmp Master Mix (Applied Biosystems) and EDTA pH 8.0 by the following program: 95°C for 10 min, 25 cycles of 96 °C for 5s, and 60 °C for 4 min. STA-

393 cDNA was then cleaned up by an Exonuclease I treatment (New England Biolabs, Beverly,
394 MA). The resulting cDNA product was diluted 5-fold in DNA Suspension Buffer (TEKnova),
395 loaded into the Fluidigm 96.96 Dynamic Array following Fluidigm protocols
396 (https://www.fluidigm.com/documents), and assayed for 88 DvH genes by quantitative
397 PCR (qPCR) using Sso Fast EvaGreen Supermix (Bio-Rad Laboratories) with ROX passive
398 reference dye by the following program: 95 °C 60 seconds, 40 cycles of 96 °C for 5 seconds
399 and 60 °C for 20 seconds, and melting curve analysis from 60-95 °C.
400 For each strain (WT or DVU0744::Tn5), condition (SR or ST), and time-point
401 (transfer 1 or 3) (8 samples total), we measured amplification of 88 DvH genes in the
402 following: single cells with RT (n=80), single cells without RT as genomic DNA background
403 controls (n=6), positive control of 10.6 pg purified DvH RNA with RT (n=4), positive control
404 of 10.6 pg purified DvH RNA without RT (n=2), and no template controls (n=4).
405
406 *Single cell analysis.* BioMark Real-Time PCR Analysis software (Fluidigm Inc. South San
407 Francisco, CA) was used to analyze amplification and melting curves for each single cell and
408 control for all 88 assays. Cycle of quantification (C_q) thresholds were set using the
409 AutoGlobal method and the baseline correction method used was Linear Derivative. Raw
410 data for RT single cells and no RT single cells are displayed for all assays and samples in
411 Supplemental Figures 1-2.
412 Several steps to verify controls were carried out as follows. Positive controls treated
413 with RT and without RT (no RT) were used to confirm that reactions were not
414 contaminated, not amplifying non-specifically, and not cross-reacting with other assays
415 (Supplemental Figure 3). Excluded from further analyses were cells or controls with

atypical amplification curves or melting temperatures (T_m) that varied significantly from the positive control in a Student's t-test (Supplemental Figures 4-11). Relative quantity of molecules (RQ) was calculated from each C_q value for more intuitive analysis (Ståhlberg et al. 2013) using the equation: $RQ = 2^{(Cqcutoff - Cq)}$. We used data collected from "no RT" single cells to set the limit of detection rather than setting an arbitrary limit of detection as in Ståhlberg et al. 2013. Cqcutoff was set to the median of "no RT" single cell controls to yield an limit of detection (LOD) at RQ=1, or $\log_2 RQ=0$, or 1 molecule present in the reaction, which is what we expect for each single copy gene amplified from genomic DNA in the "no RT" single cell controls. Assays from RT-treated single cells that did not amplify (C_q = NA), or amplified at very high Cq, were set to RQ = 0.5 molecules, or log2RQ=-1, thus below the LOD (RQ = 1 molecule) following methods described in Ståhlberg et al. (2013). All analyzed data is provided in Supplemental Table 2. Further analysis was only performed on single cells that amplified in both 16S and 23S rRNA assays (Supplemental Figure 12). Finally, the expression of the most highly expressed genes among single cells was compared to population-level expression measured by RNA-Seq (Supplemental Figure 13).

Principal component analysis (PCA). PCA was used to compute and visualize the distances between single cells based on their expression in the target genes (excluding signals from 16S and 23S rRNA genes). To limit the noise signal near the limit of detection for gene expression, PCA was completed with a subset of highly expressed genes. The list of highly expressed genes was a set of the 10 most highly expressed genes that was gathered from each population. These lists were combined into a set of 13 unique genes for further examination. In addition, we used PCA to compute and visualize distances between genes

based on their expression across single cells. The PCA of genes was conducted separately for each population of single cells. PCAs were completed in R using the function *princomp*.

Bean plots and violin plots. Beanplots and violin plots were used to visualize the distribution of gene expression levels and numbers of expressed genes in the different cell populations. These programs were implemented in R using *beanplot* (Kampstra 2008) and *vioplot* (Hintze & Nelson 1998).

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Author contributions

AWT, ST, DAS, and NB designed the experiments. AWT carried out the cell sorting. AWT and CEA carried out the cDNA synthesis and qRT-PCR. AWT, ALGL, and AR completed analysis. AWT wrote the manuscript with contributions from all authors.

462

463 **Conflict of interest**

464 The authors declare no conflict of interest.

465

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