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Single-cell analysis reveals gene-expression heterogeneity in syntrophic dual-culture of *Desulfovibrio vulgaris* with *Methanosarcina barkeri*

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Microbial syntrophic metabolism has been well accepted as the heart of how methanogenic and other anaerobic microbial communities function. In this work, we applied a single-cell RT-qPCR approach to reveal gene-expression heterogeneity in a model syntrophic system of *Desulfovibrio vulgaris* and *Methanosarcina barkeri*, as compared with the *D. vulgaris* monoculture. Using the optimized primers and single-cell analytical protocol, we quantitatively determine gene-expression levels of 6 selected target genes in each of the 120 single cells of *D. vulgaris* isolated from its monoculture and dual-culture with *M. barkeri*. The results demonstrated very significant cell-to-cell gene-expression heterogeneity for the selected *D. vulgaris* genes in both the monoculture and the syntrophic dual-culture. Interestingly, no obvious increase in gene-expression heterogeneity for the selected genes was observed for the syntrophic dual-culture when compared with its monoculture, although the community structure and cell-cell interactions have become more complicated in the syntrophic dual-culture. In addition, the single-cell RT-qPCR analysis also provided further evidence that the gene cluster (DVU0148-DVU0150) may be involved syntrophic metabolism between *D. vulgaris* and *M. barkeri*. Finally, the study validated that single-cell RT-qPCR analysis could be a valuable tool in deciphering gene functions and metabolism in mixed-cultured microbial communities.

Dissimilatory sulfate-reducing prokaryotes (SRB) are a diverse group of anaerobic bacteria that are widespread in nature that play an essential role in the global cycling of carbon and sulfur^{1,2,3}. The SRB are known to have two major lifestyles: sulfidogenic and syntrophic metabolism. In the presence of sulfate, SRB use it as the terminal electron acceptor during the oxidation of various products of primary fermentations and oxidize them to CO₂^{2,4}. When sulfate is depleted, SRB in general ferment organic acids and alcohols, producing hydrogen, acetate and carbon dioxide, and rely on hydrogen- and acetate-scavenging methanogens to convert these compounds to methane^{5,6}. This working relationship of SRB with methanogens is known as 'syntrophy' and has been considered as the heart of how methanogenic and other anaerobic microbial communities function⁷. A model syntrophic interaction involves lactate oxidation by *Desulfovibrio vulgaris* to produce acetate, CO₂, and H₂ as products, which the methanogen, *Methanosarcina barkeri*, can then further convert to CH₄⁸. The removal of H₂ by *M. barkeri* provides a thermodynamically favorable condition (*i.e.*, low H₂ concentration) for the continued oxidation of lactate by *D. vulgaris*^{3,8}. The advantage of having two different metabolic capabilities is that it raises the chances of survival of SRB in environments where electron acceptors could become depleted³. While the physiology of the symbiotic relationship of SRB and methanogens has been studied for several decades years^{3,9}, relatively little is known about the genes and their expression dynamics associated with the syntrophic interactions, partially due to the lack of suitable methodologies for measurements of biological properties in mixed-culture systems in the past. To address the issue, a transcriptomic analysis approach, which is able to distinguish transcripts from each of two species participated in the syntrophic relationship, was recently employed to compare the gene expression profiles of *D. vulgaris* in sulfate-limited monocultures and in syntrophic dual-cultures with a hydrogenotrophic methanogen *Methanococcus maripaludis*¹⁰, and of *D. vulgaris* during its metabolic shift from syntrophic growth with *M. barkeri* to sulfidogenic



growth¹¹. The results showed that between the two lifestyles, several hundred genes including those encoding ATPase, hydrogenases and high-molecular-weight cytochrome were differentially regulated, suggesting their potential roles to syntrophic growth relationship in *D. vulgaris*^{11,12}. Interestingly, a gene cluster encoding several functionally unknown lipoproteins and membrane-bound proteins (DVU0145 to DVU0150) was found up-regulated in syntrophic dual-cultures when compared with the monocultures¹⁰, and down-regulated when *D. vulgaris* cells were shifted from syntrophic to sulfidogenic metabolism¹¹, suggesting they may be involved in syntrophic metabolism. However, so far no further investigation on these genes have been conducted.

Single-cell microbiology has attracted significant attention as more evidence suggested that even isogenic populations of microorganisms could have substantial cell-to-cell heterogeneity at both cellular and molecular levels^{12–17}. For example, a RT-qPCR analysis of individual cells from the identical *Escherichia coli* population showed that the expression level of highly expressed the 16S rRNA gene could vary up to ~32-fold between single cells of the same population¹⁸. In addition to micro-scale environmental differences, it is currently known that gene-expression stochasticity, or noise, once amplified through generations, could eventually generate heterogeneity at the cellular level in a clonal bacterial population^{17,19,20}. The significant gene-expression heterogeneity observed for a microbial population suggests that by simply harvesting and analyzing mRNA or proteins from whole populations, it may not be able to capture the unique patterns of gene expression related to distinct functional subpopulations. When it comes to mixed cultures, single-cell based analysis may be more valuable as the heterogeneity within a mixed population could be even higher as different types of cells with distinct metabolic profiles, interaction and stress responses, are co-cultivated within one culture²¹. Although single-cell genomics has been applied to a handful of symbiotic systems, including bacterial symbionts of marine sponges, insects (grasshoppers, termites)²², to our knowledge, the single-cell based gene-expression analysis has so far not been applied to any syntrophic microbial system, and the dynamics of gene expression and metabolic status in cells of syntrophic mixed cultures remains unclear.

Due to their small size, tough cell walls, short half-life of the bacterial mRNA as compared with those from eukaryotic cells, and low content of mRNA, the gene expression quantitation in single bacterial cells has been challenging. We recently developed a two-step protocol to measure gene expression level in single bacterial cells using real-time reverse-transcription quantitative PCR (RT-qPCR) approach, and has demonstrated the method is sensitive enough not only for measuring cellular responses at the single-cell level, but also for revealing gene expression heterogeneity among bacterial cells^{18,23}. To further decipher metabolic and regulatory mechanisms associated with the syntrophic metabolism in *D. vulgaris* - *M. barkeri* system¹¹, we employed the single-cell RT-qPCR method to compare the gene expression dynamics of selected target genes in *D. vulgaris* grown in monoculture and in dual-culture with *M. barkeri*, with the major goals to reveal the gene-expression heterogeneity in *D. vulgaris* populations grown under two different conditions (*i.e.*, sulfate-reducing and syntrophic), and to seek further confirmation of roles of the selected genes in syntrophic metabolism at a single-cell resolution. The study provided the first quantitative description for the gene-expression heterogeneity in the *D. vulgaris* - *M. barkeri* syntrophic dual-culture.

Results and Discussion

Growth of *D. vulgaris* in monoculture and syntrophic dual-culture. To accurately compare gene expression in the *D. vulgaris* grown in monoculture and syntrophic dual-culture, a quantitative method to determine growth of *D. vulgaris* in dual-culture was employed. Using

the lactate dehydrogenase encoding gene (DVU0600) that is not present in *M. barkeri* as an index²⁴, we used a pair of lactate dehydrogenase specific primers in a quantitative PCR using the *D. vulgaris* chromosomal DNA as template, and established relationship between cell numbers of *D. vulgaris* and C_T values of qPCR. The results showed that within a range of 10^5 to 10^8 *D. vulgaris* cells, a linear relationship between cell numbers of *D. vulgaris* and C_T values of qPCR can be achieved (Suppl. Fig. S1). In addition, the reliability of this method was also independently confirmed using cell counting under microscope (data not shown). Using the method, *D. vulgaris* growth-time courses in monoculture and syntrophic dual-culture were determined (Fig. 1AC). Visual examination of *D. vulgaris* monoculture and *D. vulgaris* - *M. barkeri* dual-culture was also conducted under microscope to exclude any possible contamination (Fig. 1BD).

To compare gene expression between sulfidogenic and syntrophic metabolism, *D. vulgaris* cells at middle-exponential and stationary phases from both monoculture and dual-culture were collected (*i.e.*, 41 and 65 h for monoculture, and 11 and 23 h for syntrophic dual-culture, respectively) (Fig. 1). The cells obtained from the cultures were suspended immediately in RNA protectant RNALater solution (Ambion, Carlsbad, CA) to keep their gene expression profiles intact, and then subjected to single-cell isolation. In the study, 30 individual single *D. vulgaris* cells were randomly isolated from each sample using a single-cell manipulator, and then used for the RT-qPCR analysis. For the dual-culture, no physical association between *D. vulgaris* and *M. barkeri* was observed, so the isolation of the *D. vulgaris* single cells will not affect the metabolic states of the cells. The single-cell isolation process for 30 cells can typically be finished within 20 min, which ensured no significant change of gene expression profiles²⁵.

Primer optimization for target genes of single cell RT-qPCR. Our previous single-cell studies have found that even with optimized design using various commercial or public software, only a fraction of primers functioning well for bulk cells can be successfully applied to single-cell analysis^{18,23}, so a process of primer selection and optimization need to be implemented at single-cell level. Since the major goal of this study is to determine gene-expression heterogeneity difference between *D. vulgaris* monoculture and *D. vulgaris* - *M. barkeri* syntrophic dual-culture, 6 target genes (*i.e.*, DVU0145: response regulator, DVU0148: lipoprotein, DVU0149: hypothetical protein, DVU0533: Hmc operon protein 4, DVU1922: periplasmic [NiFe] hydrogenase large subunit, and DVU2402: heterodisulfide reductase subunit A) possibly related to syntrophic growth and 3 potential internal reference genes (*i.e.*, Dv16SA: the 16S ribosomal RNA, DVU1090: recombinase A, and DVU0600: L-lactate dehydrogenase) were selected (Table 1). For the target genes, DVU0148 and DVU0149 are located in the DVU0146-DVU0150 gene cluster currently without any functional annotation, although previous studies have found they were differentially regulated between sulfidogenic and syntrophic life styles of *D. vulgaris*^{10,11,26}; meanwhile none of genes in the cluster were differentially regulated under various *D. vulgaris* pure culture conditions tested^{27,28}. DVU0145 encodes a response regulator immediately upstream of the DVU0146-DVU0150 cluster, with the same transcriptional direction, probably involved in regulation of the DVU0146-DVU0150 gene cluster²⁹. For DVU0533 encoding high-molecular-weight cytochrome (Hmc) and DVU1922 encoding periplasmic hydrogenases (Hyn), a previous study has found that they were among genes with the largest increases in expression in *D. vulgaris* syntrophic metabolism¹⁰. In addition, early studies have showed that DVU2402 was up-regulated during the *D. vulgaris* syntrophic metabolism^{10,11}. Based on these early results, DVU0145, DVU0148, DVU0149, DVU0533, DVU1922, and DVU2402 were selected for single-cell analysis in this study.

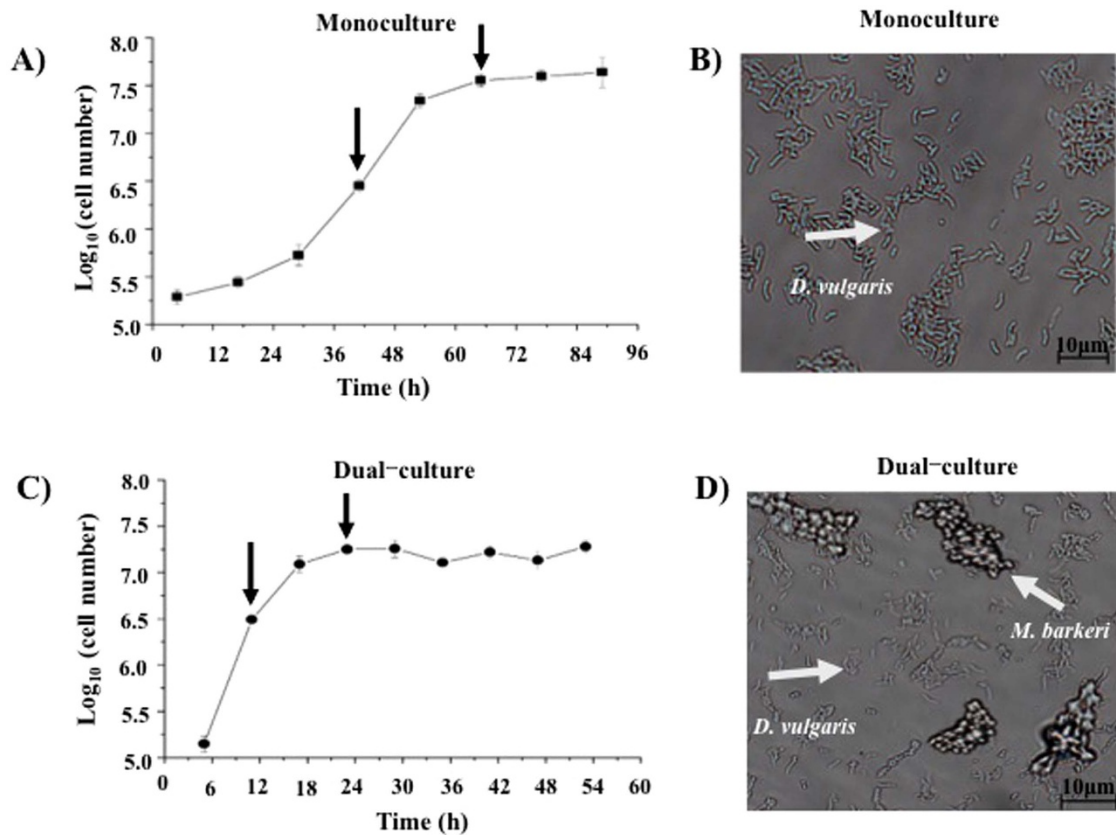


Figure 1 | Growth curves of *D. vulgaris* in monoculture (A) and syntrophic dual-culture with *M. barkeri* (C). In addition, the microscopic photos of monoculture (B) and syntrophic dual-culture with *M. barkeri* (D) were also provided. The arrows in plot (A) and (C) indicated the sampling time points for single-cell RT-qPCR analysis. In plot (B) and (D), *D. vulgaris* and *M. barkeri* were indicated, respectively.

For each gene, 4–9 sets of primers were designed and evaluated first using cDNA template synthesized from bulk-cell RNA (*i.e.*, 10^4 – 10^6 cells), and then using cDNA from single cells according to the evaluation criteria described before²³ (Suppl. Table S1). To achieve high-efficient amplification in RT-qPCR, we used similar criteria described in previous works to design the primers^{18,23}: *i*) The length of each primer was 18–24 bp; *ii*) The melting temperature of each primer pair was similar (a difference of 5°C or less); *iii*) Since GC-rich primers could form a stable heterozygote and AT-rich primers could reduce melting temperature of the well-matched heterozy-

gotes, the content of GC in each primer was about 50%; *iv*) The length of each amplified fragment should be about 100–200 bp.

A total of 51 pairs of PCR primers were evaluated for 6 target genes and 3 internal reference genes. The criteria used to select single-cell primers are: *i*) single target peak in the melting curve; *ii*) the smallest threshold cycle (C_T) number among all successful primer sets for the same target gene; *iii*) the smallest variation between analytical replicates of RT-qPCR analysis. In all study, we used three analytical replicates for each single cell. The efforts allowed identification of one set of optimized primers for each gene target (Table 1).

Table 1 | Optimized primers for target genes for single-cell analysis

Gene ID	Gene name	Annotation	Primer names	Sequence (5'→3')
DVU1922	<i>hynA-1</i>	Periplasmic [NiFe] hydrogenase large subunit, isozyme 1	DVU1922G-fw DVU1922G-rv	AAGCGCATCCAGGAATTCAC TGGTGCCACCGTACTGTGTC
DVU0533		Hmc operon protein 4	DVU0533B-fw DVU0533B-rv	CTACACCCTGCACGAGTTCATG GGAAGAGCCAGTAACCCACGTA
DVU2402	<i>hdrA</i>	Heterodisulfide reductase subunit A	DVU2402C-fw DVU2402C-rv	GGCGAAACTCGACAAGACCTT GATGTTACCCGTGAAGTTGCC
DVU0145		Response regulator	DVU0145A-fw DVU0145A-rv	TCATCATCCTGAGCGGACA GCGTTCGTAGACGTTTTTCCA
DVU0148		Lipoprotein	DVU0148D-fw DVU0148D-rv	TTCTCGATGCCCGATCATACA TCATGAAGCGGAGCGGATACAC
DVU0149		hypothetical protein	DVU0149A-fw DVU0149A-rv	CAAGTTCGGCAACGTCGACTAC AGATCCAGCGAACGACCTTCTC
DVU0600	<i>ldh</i>	L-lactate dehydrogenase	DVU0600H-fw DVU0600H-rv	ATGAACAGGATAGCCGTCA GGCGTCTATGAGAACGATG
DVU1090	<i>recA</i>	Recombinase A	DVU1090H-fw DVU1090H-rv	GCCGTCTATTCATCAACCAG TCCATACGGACGGAACCTGTAGA
Dv16SA	<i>rrsA</i>	16S ribosomal RNA	Dv16SAC-fw Dv16SAC-rv	CAACCCCTATTGCCAGTTGCT GCCATGATGACTTGACGTCGT



Selection of internal reference gene. In order to ensure that the gene expression across different conditions or analytical platforms is quantitatively comparable, expression measurements need to be normalized against an internal reference gene in RT-PCR analyses^{26,30}. Although successful application of several internal reference genes has been demonstrated in bulk-cell-based RT-qPCR analyses in various microbes, so far limited information is available regarding the constant expression of these internal reference genes across single cells^{31–34}. In addition, early studies suggested that they could also be variable among single cells³⁵. In a previous study, Shi et al. (2013) evaluated the expression variation of three possible internal reference genes, the tubulin gene, the 18S rRNA gene, and the actin gene in 12 single cells for a marine diatom *Thalassiosira pseudonana*, and the result showed that even for the 18S rRNA and tubulin genes, which were widely used as internal reference genes in various bulk-cell-based RT-qPCR analyses, significant cell-cell heterogeneity of their gene expression levels existed in *T. pseudonana* single cells²³. So far no single-cell RT-qPCR analysis has been reported for *D. vulgaris*, thus no information is available for internal reference gene. As the proper use of the internal reference gene is the key to a successful RT-qPCR gene expression analysis³², we first evaluated the performance of several possible candidates as internal reference genes in single-cell analysis. Based on previous RT-PCR studies in bulk cells of *D. vulgaris* and other similar species, the 16S ribosomal RNA gene (Dv16SA)^{11,36}, the L-lactate dehydrogenase gene (DVU0600, *ldh*), and the recombinase A gene (DVU1090, *recA*)^{37,38}, were selected as candidates of internal reference genes for further evaluation. Two sets of single cells, each

with 13 single cells, were randomly isolated from both *D. vulgaris* monoculture and dual-culture, respectively, and subjected to gene-expression analysis for the three candidates of internal reference genes. The standard deviations (SD) of the C_T values were calculated using the OriginPro 8.0 software. The results showed that the standard deviations (SD) of the C_T values were 0.98, 0.52 and 0.66 cycles among 13 single cells from the monoculture, and 0.88, 0.74 and 0.49 cycles among 13 single cells from the dual-culture, respectively (Fig. 2), and the mean C_T values were 29.31, 33.66 and 31.78 cycles among 13 single cells from the monoculture, and 27.60, 33.00 and 32.47 cycles among 13 single cells from the dual-culture, for the Dv16SA, DVU0600 and DVU1090 genes, respectively. Although slightly different between monoculture and dual-cultures, DVU1090 (*recA*) presented a relatively low variation of SD among single cells and low difference of mean C_T values between two growth modes, which is consistent with the previous publications for its stable expression in bulk cells^{37,39}, and was thus selected as an internal reference gene for the further RT-qPCR analysis in this study. In various bulk-cell based studies, Dv16SA was often considered to be a good internal reference gene for gene expression analysis for *D. vulgaris*^{11,34}; however, our single-cell analysis showed that significant heterogeneity existed for the Dv16SA gene, and it may not function well as an internal reference gene for single-cell based analysis. The cause and biological significance that certain genes are more variable than others across single cells may worth further investigation.

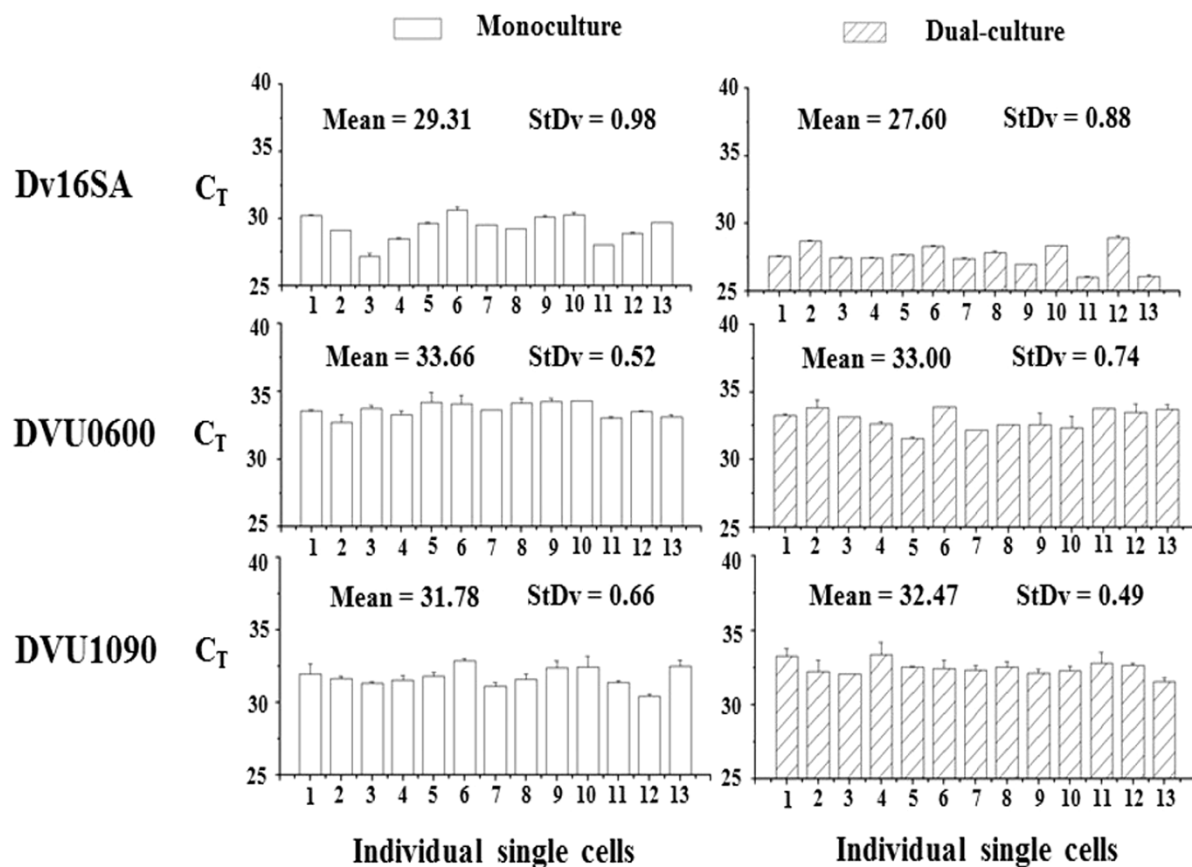


Figure 2 | Evaluation of three internal reference candidates in monoculture and syntrophic dual-culture. C_T is the qPCR quantification cycle, the fractional cycle number where fluorescence increases above the threshold. 13 cells from monoculture and 13 cells from syntrophic dual-culture were used to evaluate the consistency of the internal reference genes. The standard deviations (SD) across single cells were calculated using the OriginPro 8.0 software. Mean C_T values and SD values calculated using 13 single cells from each of the two conditions (*i.e.*, mono- or dual-cultures) were shown above the plots.



Gene-expression dynamics in *D. vulgaris* population in mono-culture and dual-culture. To uncover gene-expression dynamics at single-cell level, we isolated 30 individual cells from each sample (*i.e.*, mono- and dual cultures, exponential and stationary phases), and analyzed 6 target genes and one internal reference gene (*i.e.*, DVU0145, DVU0148, DVU0149, DVU0533, DVU1922, DVU2402 and DVU1090) in each of the single cells. Each gene was analyzed with three analytical replicates. The ΔC_T method was used to calculate the relative expression of each gene against the internal reference gene *recA*.

A quality control was manually conducted for a total 2,520 reactions of qPCR analysis (*i.e.*, 30 cells \times 4 conditions \times 7 genes \times 3 analytical replicates). The results showed a qPCR success rate of approximately 91%, after removing reactions with large variations between analytical triplicates (*i.e.*, SD > 0.5), and/or with wrong amplification peaks in the melting curves, giving a total of 28, 27, 28 and 26 cells out of 30 initially isolated cells with all gene successfully analyzed in monoculture at exponential and stationary phase, and dual-culture at exponential and stationary phases, respectively. The reproducibility analysis showed relatively small technical variation for all genes under all conditions, which were 0.147 cycle (0.441% of average C_T values), 0.145 cycle (0.436%), 0.155 cycle (0.463%), 0.197 cycle (0.604%), 0.184 cycle (0.559%), and 0.181 cycle (0.540%) for DVU0145, DVU0148, DVU0149, DVU0533, DVU1922, and DVU2402 genes, respectively (Table 2). In addition, comparison of the technical variation of the same genes across different samples showed relatively smaller SD for cells of exponential phases, and slightly greater SD for cells of stationary phases, which may be reasonably due to the decreasing metabolic activity and even RNA contents in cells of stationary phase. Moreover, no difference in terms of qPCR technical variation was observed between mono- and dual-cultures at both the exponential and stationary phases. Overall, the quality control analysis demonstrated that the single-cell RT-qPCR protocol used in this study was robust and able to generate reproducible data for biological investigation.

With the qualified single-cell RT-PCR data, the first question we attempted to address was how much heterogeneity existed in the *D. vulgaris* cultures. To do so, we plotted distribution according to relative gene expression levels in each of single cells for all target *D. vulgaris* genes under in both monoculture and dual-culture (Fig. 3–6). The results showed that for all target genes under all tested condition, significant gene-expression heterogeneity existed, as a wide distribution along the gene-expression level was clearly observed. For example, gene-expression level varied as much as 50-fold in different single cells for DVU2402 at exponential phase under syntrophic dual-culture condition (Fig. 3), and as much as 40-fold in different single cells for DVU0533 at stationary phase under monoculture condition, respectively (Fig. 4). The results were consistent with the previous study with *T. pseudonana* under various stress conditions²³, suggesting the gene-expression heterogeneity across individual single cells could be a common phenomenon in

microbial population. In addition, the genes were also different in terms of the degree of heterogeneity, as a comparison found that among all 6 genes, DVU0533 tended to have the largest heterogeneity under all growth conditions, while DVU0149 had the smallest heterogeneity under all growth modes (Fig. 3–6). While the different heterogeneities among genes may be partially related to expression levels of the genes and possible measurement variations, its biological significance may worth further investigation.

The second question we attempted to address was whether an increased complexity of community structure in the *D. vulgaris* – *M. barkeri* dual-culture, accompanying by increased complexity in terms of micro-environments and the cell-cell communication from among different *D. vulgaris* cells to among *D. vulgaris* and *M. barkeri* cells, could result in an increased gene-expression heterogeneity in *D. vulgaris*. The degree of gene-expression heterogeneity can be defined by the range between cells with the lowest and highest gene expression level. However, when we compared gene-expression heterogeneity of all 6 genes between the monoculture and dual-culture at both exponential and stationary phases, the results showed no obvious difference in terms of gene-expression heterogeneity between monoculture and dual-culture at both growth phases (Fig. 3–4), suggesting that increase of complexity of community structure in the *D. vulgaris* – *M. barkeri* dual-culture didn't result in change of heterogeneity for the 6 target genes analyzed. While this result seemed unexpected, one possible explanation is that the 6 target genes used in this study were potentially related to syntrophic metabolism, and their expression may be enhanced in all single cells during syntrophic metabolism.

With the qualified single-cell data from four conditions (*i.e.*, mono- and dual-cultures at exponential and stationary phases), the third question we attempted to address was whether the gene-expression distribution of the selected genes varied between different conditions. To do so, we established four pairs of comparisons for 6 target genes (*i.e.*, mono- vs. dual-culture at exponential phase, mono- vs. dual-culture at stationary phase, exponential vs. stationary in dual-culture, and exponential vs. stationary in monoculture), with an emphasis to the first two pairs of comparisons that may reveal genes functionally related to syntrophic metabolism. The similarities of the two distributions of any given gene were evaluated for each pair of comparisons, indicating by *p*-values determined by using the nonparametric two-sample Kolmogorov-Smirnov test between two conditions. In addition, in the box plots, similarities of gene expression data of two conditions for any given gene were evaluated, indicating by *p*-values calculated by means of the two-tailed nonparametric Mann-Whitney statistical significance test. The comparison of mono- vs. dual-culture at exponential phase was presented in Fig. 3. The results showed that the distributions of all 6 genes were shifted towards right side in the plots, representing an up-regulation of gene expression in single cells of the *D. vulgaris* dual-culture, suggesting that these genes may be related to syntrophic metabolism, consistent with the conclusion from previous bulk-cells based studies^{10,11,36}. In addition, nonparametric two-sample Mann-Whitney

Table 2 | Average standard deviation values among 30 single-cells for six target genes under four growth conditions

Growth Conditions	DVU0145		DVU0148		DVU0149		DVU0533		DVU1922		DVU2402	
	SD-Mean	% of SD/ C_T -Mean	SD-Mean	% of SD/ C_T -Mean	SD-Mean	% of SD/ C_T -Mean	SD-Mean	% of SD/ C_T -Mean	SD-Mean	% of SD/ C_T -Mean	SD-Mean	% of SD/ C_T -Mean
DE	0.183	0.555	0.105	0.316	0.093	0.281	0.213	0.659	0.197	0.607	0.147	0.442
ME	0.104	0.314	0.142	0.432	0.139	0.418	0.199	0.622	0.181	0.54	0.181	0.541
DS	0.170	0.512	0.173	0.522	0.147	0.444	0.19	0.587	0.167	0.515	0.166	0.502
MS	0.158	0.468	0.164	0.485	0.227	0.663	0.186	0.554	0.194	0.575	0.218	0.638
All Conditions	0.147	0.441	0.145	0.436	0.155	0.463	0.197	0.604	0.184	0.559	0.181	0.540

ME and DE are abbreviations of exponential phase in monoculture and syntrophic dual-culture, respectively. MS and DS are abbreviations of stationary phase in monoculture and syntrophic dual-culture, respectively.

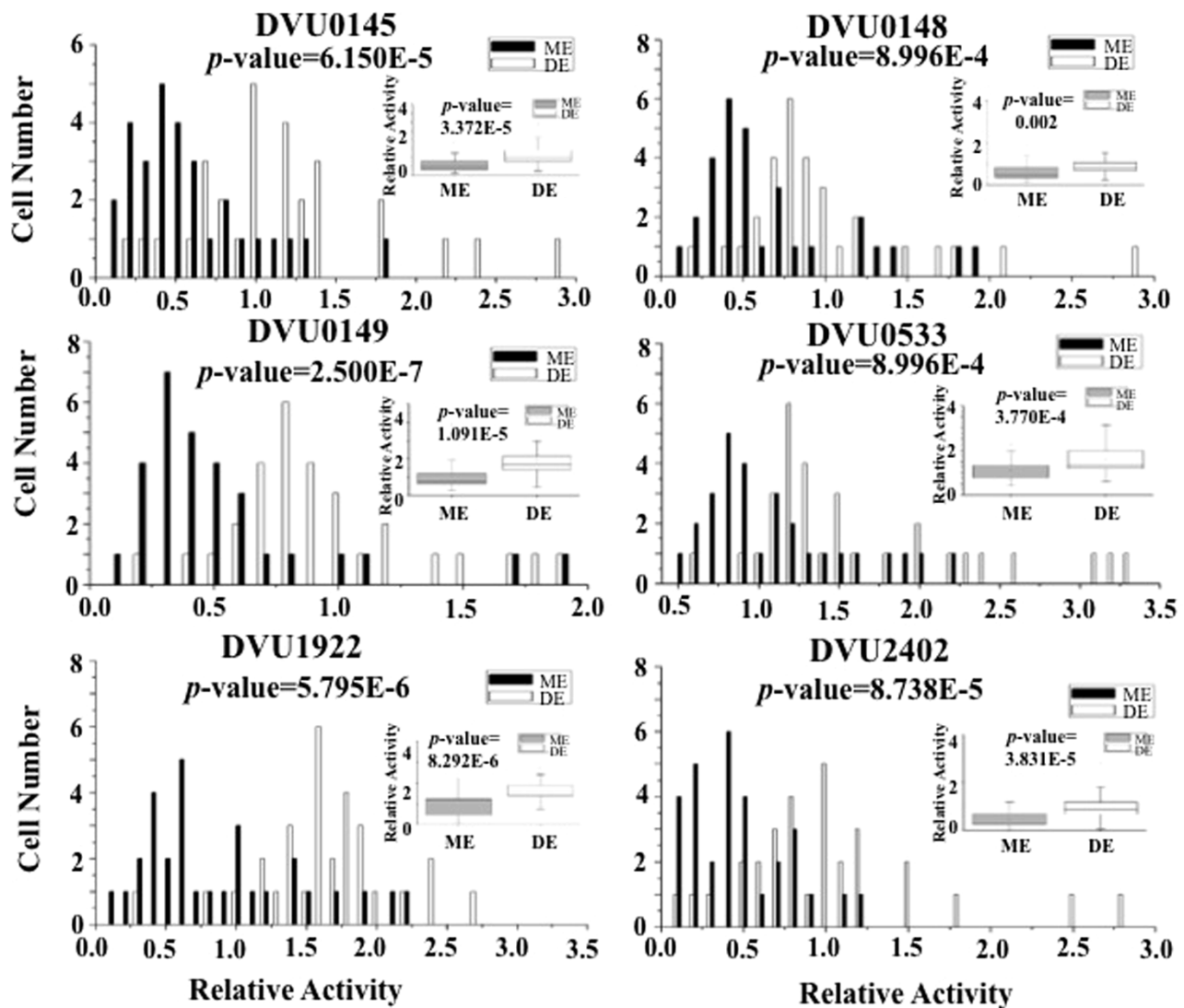


Figure 3 | Gene-expression distributions of target genes at exponential phase in monoculture and syntrophic dual-culture. ME and DE are abbreviations for exponential phase in monoculture and syntrophic dual-culture, respectively. *P*-values in the histograms were determined by using the nonparametric two-sample Kolmogorov-Smirnov test between monoculture and syntrophic dual-culture ($\alpha = 0.05$). The *x* axis shows the relative activity of a specific gene compared with the internal reference gene DVU1090 in the same cell, and the *y* axis shows the number of cells that have the same relative activity. *P*-values in the box plots were calculated by means of the two-tailed nonparametric Mann-Whitney statistical significance test between monoculture and syntrophic dual-culture. Box plots of single-cell gene expression levels and *P*-values associated with the differences between monoculture and syntrophic dual-culture. Box plot shows following statistical values: Open square – mean, solid line – median, upper and lower box lines – the 75th and 25th percentiles, respectively. Top and bottom short line – maximal and minimal values, respectively.

tests were also performed for each of the target genes between two conditions, and *p*-values in box plots were calculated to indicate the statistical significance of the difference between two sets of single cell datasets. The statistical results provided further supports for the distribution patterns described above, with *p*-values of all genes significantly lower than 0.05 (Fig. 3).

When comparing the single-cell RT-qPCR results of mono- and dual-culture at stationary phase, we found that only 4 out of 6 target genes, DVU0149, DVU0533, DVU1922 and DVU2402 were up-regulated in syntrophic metabolism, as their distributions were shifted towards right with statistical significance less than 0.05, while gene DVU0145 and DVU0148 showed no difference in terms of their distribution patterns between the two phases (Fig. 4), suggesting the two genes were functional primarily at fast-growing exponential phase^{10,11}. This conclusion was further confirmed when we compared single-cell gene-expression patterns between exponential and stationary phases in dual-culture, with the distributions of 5 genes

(i.e., DVU0145, DVU0148, DVU0149, DVU0533 and DVU1922) shifted left at stationary phase with statistical significance less than 0.05 (Fig. 5). For the DVU2402 gene, although its expression distribution shift was not so significant, with a *p*-value of 0.071, its expression level was visibly up-regulated for dual-culture compared with monoculture, with a *p*-value of 0.041 (Fig. 4), suggesting the gene may also be functioning in syntrophic dual-culture^{10,11}.

We also compared the gene-expression distribution among single cells between exponential and stationary phases in monoculture (Fig. 6). The results showed that 4 of the 6 genes (i.e., DVU0145, DVU0148, DVU0149 and DVU2402) have no differences in terms of the distribution patterns between the two phases in monoculture, with statistical significances of nonparametric two-sample Mann-Whitney tests greater than 0.05. Consistent with previous studies^{27,28,40}, the results demonstrated that these genes were not differentially regulated in *D. vulgaris* pure-culture, and the metabolic pathways that the genes involved may be important mainly only

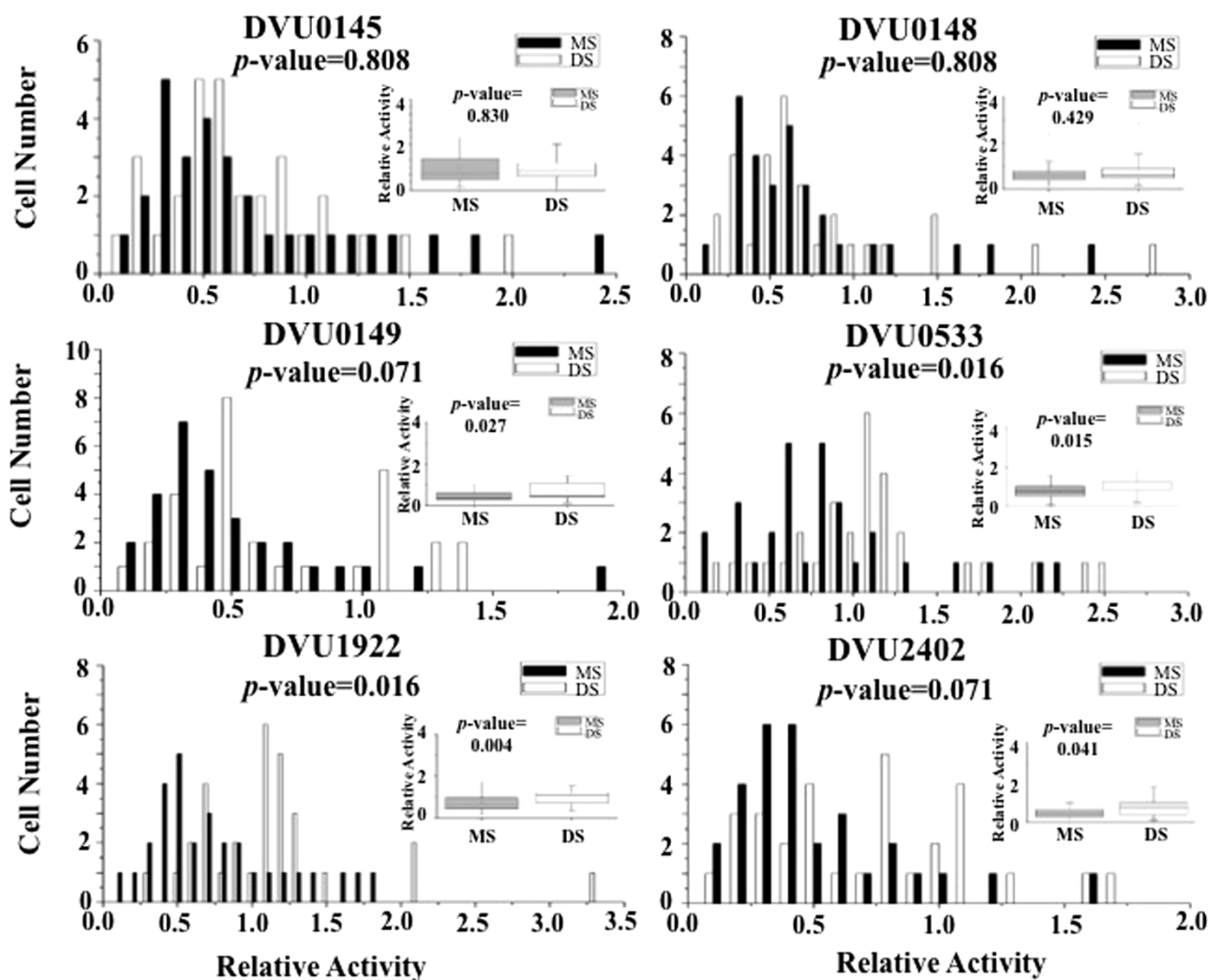


Figure 4 | Gene-expression distributions of target genes at stationary phase in monoculture and syntrophic dual-culture. MS and DS are abbreviations for stationary phase in monoculture and syntrophic dual-culture, respectively. For statistical analysis, please refer to the figure legend of Fig. 3.

during syntrophic metabolism in *D. vulgaris*. Two exceptions of this comparison were DVU1922 and DVU0533 with p -values of two-sample Mann-Whitney tests of 0.047 and 0.026, respectively, suggesting that the two genes were slightly regulated between the exponential and stationary phases in monoculture, which may be consistent with the wide functions of periplasmic hydrogenases (Hyn, DVU1922) and high-molecular-weight cytochrome (Hmc, DVU0533) in energy pathways during sulfidogenic metabolism^{27,41,42}.

Principal-component analysis of single-cell data. With the aid of sophisticated statistical tools, more intrinsic information can be extracted from single-cell-based data sets. In addition to the independence test based on gene expression distributions, principal-component analysis (PCA) could also be applied to visualize the gene-expression heterogeneity among individual single cells, and the relationship between different growth conditions and gene expression (Fig. 7). Towards these goals, two PCA score plots were generated separately, one based on genes and another on single cells. The PCA results showed the following features: *i*) all target genes can be separated in the PCA score plot (Fig. 7A), which was also confirmed by the Kruskal-Wallis ANOVA test with p -values significantly less than 0.05 (Table 3), suggesting that the single-cell RT-qPCR analysis was capable to differentiate the

independent gene-expression distribution patterns exhibited by each of the target genes^{23,43}; *ii*) DVU0145, DVU0148 and DVU0149 were clustered together in the plot (Fig. 7A), suggesting that these genes have the similar response patterns across conditions in all single cells, consistent with their genetic organization as a possible operon in the *D. vulgaris* chromosome^{11,29}; *iii*) DVU2402 was more close to the DVU0145-DVU0148-DVU0149 cluster than DVU1922 and DVU0533 in the score plot (Fig. 7A), suggesting they shared a high degree of similarity in terms of metabolic responses under the tested growth conditions; however, it remains unknown how DVU2402 functions together with the gene cluster, which may worth further investigation; *iv*) in the PCA plot based on single cells (Fig. 7B), at exponential phase, single cells from monoculture were well separated from those of dual-culture; while at stationary phase, more overlapping were observed between single cells of monoculture and those of dual-culture, suggesting the genes were differentially functional mainly at exponential phase²⁷; *v*) single cells of monoculture at stationary phase were much closer to single cells of monoculture at exponential phase, while obvious differences can be seen between single cells of dual-culture at exponential and stationary phases (Fig. 7B), suggesting the genes were not differentially regulated in monoculture; *vi*) single cells of dual-culture at stationary phase were more close to those of monoculture, although single cells of dual-culture from

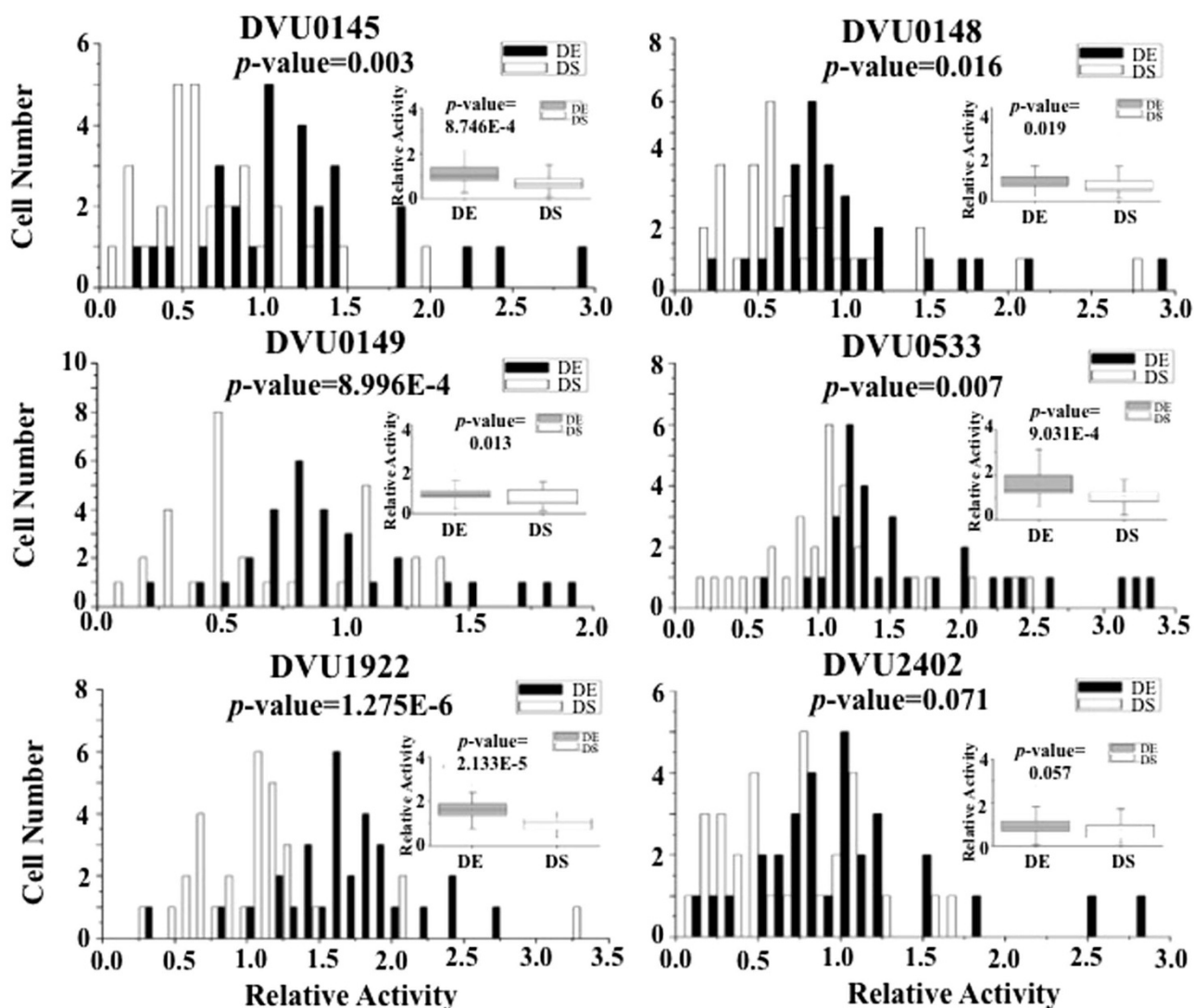


Figure 5 | Gene expression distributions of target genes at exponential phase and stationary phase in syntrophic dual-culture. DE and DS are abbreviations for exponential phase and stationary phase in syntrophic dual-culture, respectively. For statistical analysis, please refer to the figure legend of Fig. 3.

exponential phase were well separated from other three conditions (Fig. 7B), suggesting that at stationary phase, the expression levels of target genes in dual-culture were almost down-regulated to expression levels similar to those in monoculture; *vii*) finally, the means C_T of all single cells of each conditions were also calculated and plotted in the PCA plot, and the results showed that means of dual-culture at exponential and stationary phases were well separated, while no difference can be seen between exponential and stationary phase in monoculture.

Using the single-cell RT-qPCR analysis to compare gene expression heterogeneity of selected target genes in *D. vulgaris* grown in monoculture and in dual-culture with *M. barkeri*, we aimed to reveal gene-expression heterogeneity in *D. vulgaris* populations under different growth modes. The result showed significant cell-to-cell gene-expression heterogeneity for the selected *D. vulgaris* genes in both the monoculture and dual-culture. However, no increase of gene-expression heterogeneity was observed for *D. vulgaris* in the syntrophic dual-culture when compared with its monoculture, even the community structure and cell-cell interactions become more complicated in the syntrophic dual-culture. In addition, we provided further evidence at single-cell level that the gene cluster (DVU0148-DVU0150) were up-regulated in the *D. vulgaris* dual-culture when compared with its monoculture, and may be involved in syntrophic

metabolism between *D. vulgaris* and *M. barkeri*. Moreover, by comparing the gene-expression distributions under different metabolism modes, we showed evidences that the target genes (DVU0533, DVU1922, DVU2402) could also be involved in syntrophic metabolism. Finally, as one proof of concept study of microbial syntrophic metabolism, our results demonstrated that single-cell based gene expression analysis could be a valuable tool in deciphering gene functions and microbial metabolism in various microbial mixed-culture systems.

Methods

Microorganisms. *Desulfovibrio vulgaris* subsp. *vulgaris* strain Hildenborough DSM 644 and *Methanosarcina barkeri* strain Fusaro DSM 800 used in this study were purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany). Pure cultures of *D. vulgaris* and *M. barkeri* were maintained anaerobically routinely on sodium lactate (38 mM) plus sulfate (50 mM), and H_2 plus CO_2 (80 : 20) media, respectively, according to previous publications¹¹. Cultivation experiments were conducted in duplicate with dual-cultures of *D. vulgaris* and *M. barkeri* in 200 mL serum bottles containing 70 mL of medium, with head-space filled with a gas mixture of 10% (*v/v*) CO_2 and 90% (*v/v*) N_2 . *D. vulgaris* - *M. barkeri* dual-culture was performed in a medium containing: a basal salt solution containing (per liter) 0.5 g NH_4Cl , 0.413 g $MgCl_2 \cdot 6H_2O$, 0.25 g $CaCl_2 \cdot 2H_2O$, 2.25 g NaCl. The medium (per liter) was buffered using 0.348 g K_2HPO_4 and 0.227 g KH_2PO_4 with 1 mL of trace elements and 10 mL of a vitamin solution. *L*-cysteine (0.3 g/L) and $Na_2S \cdot 9H_2O$ (0.3 g/L) were added as reducing agents. Resazurin (1 mg/

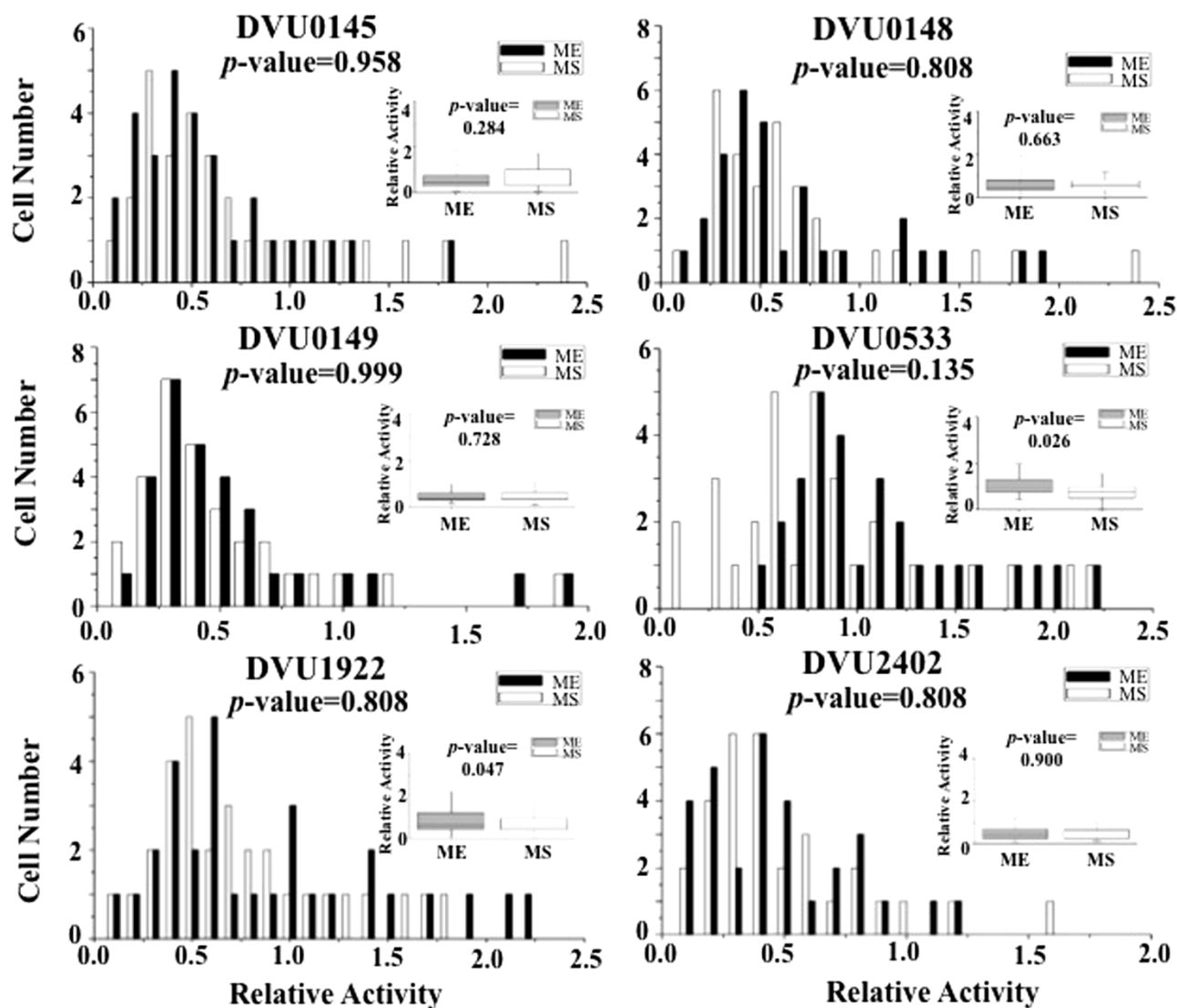


Figure 6 | Gene expression distributions of target genes at exponential phase and stationary phase in monoculture. ME and MS are abbreviations for exponential phase and stationary phase in monoculture culture, respectively. For statistical analysis, please refer to the figure legend of Fig. 3.

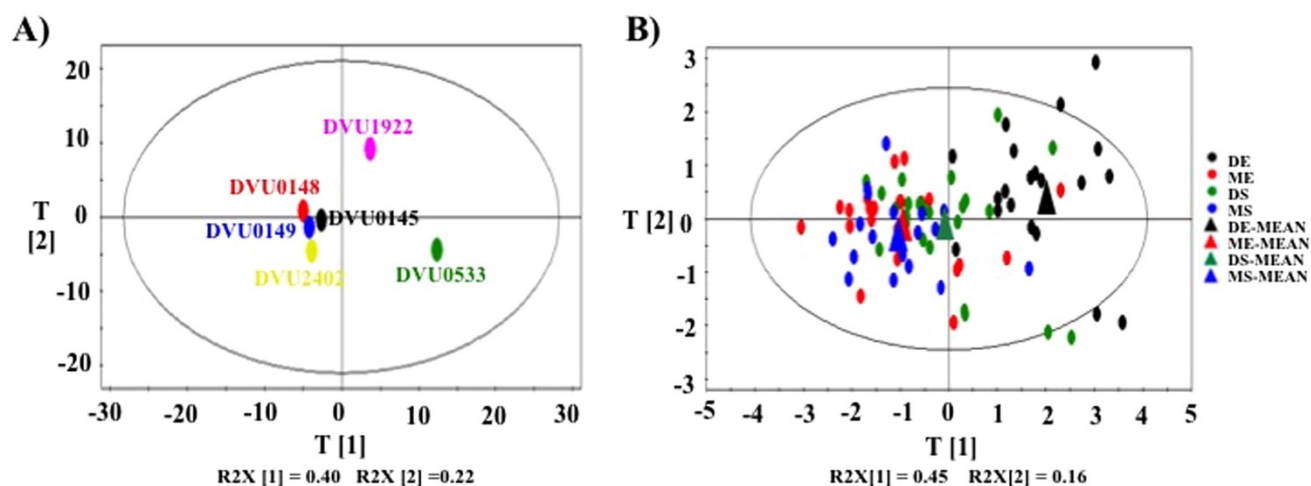


Figure 7 | Principal component analysis of target genes based on their relative expression under four growth conditions. (A) PCA score plot of six target genes using datasets containing 116 individual cells in which all target genes were successfully detected. (B) PCA score plot of all qualified single cells in which at least five target genes detected from four growth conditions. In the plot (A), each color dot represents one target gene. In the plot (B), each color dot represents one single cell of *D. vulgaris*. The means of all single-cell analyses for each condition were indicated by large triangles.



Table 3 | *P* values of Kruskal-Wallis tests at the 95% confidence level

Gene ID	<i>P</i> - value
DVU0145	1.29E-04
DVU0148	0.003
DVU0149	5.67E-06
DVU0533	2.74E-06
DVU1922	2.62E-08
DVU2402	4.01E-05

L) was added as a redox indicator. Stock solutions of K_2HPO_4 , $NaHCO_3$, *L*-cysteine, $Na_2S \cdot 9H_2O$, and the trace element and vitamin mixtures were prepared under anoxic conditions. The medium was prepared by adding salts, sodium lactate and resazurin to deionized water. Air was removed by heating, replaced by nitrogen gas and then autoclaving at 121 °C for 20 min. After sterilization, medium reservoir bottle was allowed to cool to room temperature before the remaining components were added. For monoculture, 5% inoculum of fresh *D. vulgaris* from middle-exponential with an OD_{590} of 0.4 was used, and 5% inoculum of fresh *M. barkeri* from middle-exponential with OD_{590} of 0.3 using $CO_2 + H_2$ as the carbon source; while for dual-culture, *D. vulgaris* and *M. barkeri* of equal amount (*v/v*) were independently collected by centrifugation at $6600 \times g$ for 10 min at room temperature, mixed and used as seed culture to inoculate into the dual-culture media. The dual-culture was then transferred for 6 successive passages in the same medium, with 10% inoculum (*v/v*) used for each transfer, according to Traore et al. (1983)⁴⁴. All growth experiments were conducted at 35 °C. All operation was conducted under the anaerobic condition. In the final dual-culture used for single-cell RT-qPCR analysis, we used an inoculum containing approximately 3.0×10^7 total *D. vulgaris* cells, which is the same starting cell number we used to inoculate the *D. vulgaris* monoculture.

Sampling and RNA extraction. Single-cells were picked using a micromanipulator from the diluted cell population and then were loaded into individual Eppendorf micro-tubes for the single cell analysis. In details, single cell harvesting was conducted using an Olympus single cell manipulator (Olympus Inc, Japan) with the Inverted microscope IX71 and a micromanipulator for ICSI/Injection (Narishige Group, Tokyo, Japan) with ICSI 10 micrometer diameter micropipette (Origio, Charlottesville, VA). This micromanipulator uses a piezoelectric actuated diaphragm to dispense/aspirate picoliter-level liquid through a 30- μ m capillary. Owing to the low flow rates, single cells suffer very little shear stress, which will minimize the effects on their gene expression profile. The factors that single cell of *D. vulgaris* is microscopic (0.5 ~ 1 \times 3 ~ 5 μ m) and colorless result in lower efficiency of picking cells. To overcome the problem, we used the diluted Coomassie Brilliant Blue R-250 (1 g/L) to stain cells for 1 min, which allowed better identification of cells under 100 \times oil lens (Olympus, Japan). In order to stabilize and protect cellular RNA, RNALater Stabilization Solution (Ambion, Carlsbad, CA) was added to cell culture. Thirty individual cells from each growth modes were picked. A ZR RNA MicroPrep kit (Zymo Research, Irvine, CA) was used to extract RNA from single cells, and the total RNA from single cells was eluted into an Eppendorf micro-tube with a final volume of 7 μ L.

cDNA synthesis. A SuperScript VILO cDNA synthesis kit (Invitrogen, Carlsbad, CA) was used to synthesize cDNA from single-cell total RNA. To increase the relative concentration of single-cell mRNA for cDNA synthesis preparation, the total reaction volume was designed to 10 μ L, which contained 1 μ L 10 \times Super-Script enzyme mix, 2 μ L 5 \times VILO reaction mix, and 7 μ L of eluted RNA. After cDNA synthesis, 15 μ L diethyl pyrocarbonate (DEPC)-treated water (Comwin biotech, Beijing) was added to make the final volume of 25 μ L before the mixture was used as the template for quantitative PCR analysis.

Quantitative PCR. Primers for RT-qPCR analysis were designed using Primer Express 2.0 software. To differentiate PCR products from primer dimers, we selected primers which will generate amplicons with sizes around 100 to 200 bp. qPCR was performed using Power SYBR Green PCR master mix (Invitrogen, Carlsbad, CA) on an ABI StepOne real-time PCR system for single-cell analysis (Applied Biosystems, Foster, CA). The temperature for qPCR was 10 min at 95 °C for an initial hot start, and this was followed by 40 cycles of 15 s at 95 °C for denaturing, 1 min at 60 °C for annealing and extension. There was also another melting curve analysis step that was set to be the default condition based on the real-time PCR system. For PCRs, 1 μ L of forward or reverse primer at a concentration of 5 μ M, 5 μ L of master mixture, 2 μ L DEPC-treated water, and 1 μ L cDNA were combined. Analytical triplicates of PCR analysis were performed for each gene. Reaction mixtures without cDNA templates served as negative controls. Expression levels of target genes were normalized against an internal reference gene recombinase A gene. All PCR products were sequenced by BGI (Shenzhen, China) to confirm the authenticity of the single-cell RT-qPCR analysis.

The determination of growth curves in monoculture and dual-culture using real-time qPCR. For determination of growth curves of *D. vulgaris* in syntrophic dual-

culture and monoculture, we applied a standard curve method that presents the relationship between the cell number and C_T . The initial cell number of *D. vulgaris* in monoculture was counted with a hemocytometer directly, and then diluted gradually. DNA from each diluted sample was extracted using TIANamp Bacteria DNA kit (Tiangen, Beijing, China), and then used as templates for qPCR analysis, with lactate dehydrogenase encoding gene (DVU0600) as target. The results established a standard curve between \log_{10} values of cell number and the qPCR C_T value. Using the same approach, cell number can be accurately determined using cell samples from *D. vulgaris* monoculture and syntrophic dual-culture.

Data analysis. For single-cell analysis, the resulting threshold cycle (C_T) data were normalized relative to the internal reference *recA* (recombinase A) gene using the ΔC_T method. To describe the distribution variation of single-cell gene expression levels among cells, nonparametric statistical tests that do not require normal distribution of data sets were applied⁴⁵. Kolmogorov-Smirnov, Kruskal-Wallis and Mann-Whitney analysis of variance (ANOVA) tests were also used to analyze the relationship between four different groups of RT-qPCR measurements using the OriginPro 8.0 software (OriginLab Corporation, Northampton, MA). Principal-component analysis (PCA) was conducted using the SIMCA-P⁺ 12.0 software (Umetrics, Umea, Sweden) to determine the possible control variances⁴⁶.

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Author contributions

Z.Q. performed experiments and data analysis, and wrote the manuscript. L.C. conceived, designed experiments and wrote the manuscript. G.P. helped with the data analysis. W.Z. conceived and designed experiments, analyzed the data and wrote the manuscript.

Additional information

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