

Mean and Variability in RNA Polymerase Numbers Are Correlated to the Mean but Not the Variability in Size and Composition of *Escherichia Coli* Cells

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Abstract: Cell morphology differs with cell physiology in general and with gene expression in particular. We investigate the degree to which these relationships differ with medium richness. Using *Escherichia coli* cells with fluorescently tagged β' subunits, flow cytometry, and statistical analysis, we study at the single-cell level the correlation between parameters associated to cell morphology and composition (FSC, SSC, and Width channels) and GFP tagged RNA polymerase (RNAP) levels (FITC channel). From measurements in three media differing in richness (M63, LB, and TB) and, thus, cell growth rates, we find that the mean and cell-to-cell variability in RNAP levels are correlated to the mean values of FSC, SSC, and/or Width. Further, in all growth conditions considered, RNAP levels are positively correlated to FSC, SSC, and Width at the single-cell level, with the correlation decreasing for increasing medium richness. Overall, the results suggest that the mean and cell-to-cell variability in levels of RNAP, a master regulator of gene expression, are correlated to the mean values of the parameters assessing the cellular morphology and composition, as measured by flow cytometry, but they do not correlate to the degree of variability of these parameter values.

1 INTRODUCTION

In *Escherichia coli*, the concentration of RNA polymerases (RNAP) is a key regulator of the rate of transcription (McClure, 1980, 1985; Arkin, Ross and McAdams, 1998; Kærn et al., 2005; Browning and Busby, 2016). As this concentration differs even between sisters cells (Cabrera and Jin, 2003; Bratton, Mooney and Weisshaar, 2011; Yang et al., 2014), it is an extrinsic factor for cell-to-cell variability in gene expression (Elowitz et al., 2002; Mäkelä, Kandavalli and Ribeiro, 2017).

One source of cell-to-cell variability in RNAP numbers is the noise in the chemical processes responsible for the production of RNAP (see e.g. Gillespie, 1977). Other sources include variability in cells' health, morphology, and components (Elowitz et al. 2002; Muthukrishnan et al., 2014; Oliveira et al., 2016).

Here, we investigate the degree to which the morphology and composition of the cells of a population correlate with their mean and variability in RNAP numbers. Since the environment is known

to affect the morphology and composition, we study how this correlation differs with medium richness.

For this, we use *E. coli* strain RL1314 which has GFP tagged β' subunits (Bratton, Mooney and Weisshaar, 2011). To assess both fluorescence levels as well as parameters associated to cells' morphology and composition, we use Flow cytometry. Measurements are conducted in M63, LB, and TB media, where growth rates differ. From the measurements, we collect data on the cells' green fluorescence intensity levels (a proxy for RNAP numbers), and on the cells' morphology (size) and composition. Using the data, we searched for statistically significant correlations between the RNAP levels and morphology and composition, in media differing in richness.

2 METHODS

2.1 Bacterial Cells, Chemicals, Growth Conditions, and Growth Rates

We used *E. coli* RL1314 with fluorescently (GFP) tagged β' subunits (Bratton, Mooney and Weisshaar, 2011), generously provided by Robert Landick, University of Wisconsin-Madison, U.S.A.. For cell cultures, chemicals components for Luria-Bertani (LB), terrific broth (TB) and M63 media were purchased from LabM (UK) and Sigma-Aldrich. Casamino acids and vitamins were purchased from Gibco. LB medium components are 1 g tryptone, 0.5 g yeast extract and 1 g NaCl (pH – 7.0). Meanwhile, the composition of TB medium per 100ml is 1.2 g tryptone, 2.4 g yeast extract, 0.4% glycerol and TB salts (KH₂PO₄ and K₂HPO₄). M63 medium was prepared using M63 salts supplemented with 0.4% glycerol, vitamins and 20% casamino acids.

Prior to flow cytometry, RL1314 cells were grown overnight at 30 °C with aeration and shaking in the appropriate medium, diluted 1:1000 into the fresh specific medium and allowed to grow at 37 °C at 250 rpm until an optical density at 600 nm (OD₆₀₀) of 0.4. Growth rates were measured by growth curves obtained from cells at 37°C in the appropriate medium (LB, TB and M63) with antibiotics, using a spectrophotometer (Ultrospec 10; GE Health Care). Cultures were grown overnight at 30°C with aeration and shaking at 250 rpm. Next, overnight cultures were diluted into fresh medium to an initial OD₆₀₀ of 0.01. The OD₆₀₀ values were monitored every 20 min for 3.2 h.

2.2 Flow Cytometry

For flow cytometry (FC), cells from 5 ml of bacterial culture were diluted 1:10000 into 1 ml PBS vortexed for 10 seconds and a total of 50,000 cells were tested in each run. Prior to every day experiments, the analyzer was calibrated using ACEA NovoCyte particle QC beads (Cat.No.8000004). Data was collected using an ACEA NovoCyte Flow Cytometer (ACEA Biosciences Inc., San Diego USA) equipped with a blue laser (488 nm) for excitation and the fluorescein isothiocyanate channel (FITC) (530/30 nm filter) for detecting emitted light at a flow rate of 14 μ l/minute and a core diameter of 7.7 μ M. A PMT voltage of 417 was used for FITC. To avoid background signal from particles smaller than bacteria, the detection threshold was set to 5000 in FSCH analyses.

From the flow cytometry data, we study: i) FITC, which measures the green fluorescence intensity from a cell (a proxy for the number of RNA polymerases in the cell); ii) Forward scatter (FSC), which measures the light scattered at less than 10 degrees as a cell passes through the laser beam (a proxy for cell size); iii) Side scatter (SSC), which measures the light scattered at a 90 degree angle as a cell passes through the laser beam (a proxy for cell density); and, iv) Width (W), which measures the duration of the signal, not impacted by the PMT voltage, which also correlates with cell size. Except for the Width, the FC informs on both the 'Height' (H) and 'Area' (A) of the signals. The H is the maximum peak of the signal while the A is the integration of the H measures over time.

Note that, in all conditions, we removed from the data any cell with a negative or abnormally high or low parameter value (which amounted to ~10-15% of the cells in each medium condition). This is necessary since, when ignoring one of the parameters, the correlation between this and the remaining ones cannot be obtained.

2.3 Correlations

Correlations between parameters extracted by FC are obtained by linear regressions using the least-squares fit method (95% confidence intervals), applying the Matlab function fitlm that creates a LinearModel object. We obtain the coefficient of determination (R^2) of the fitted regression line for each case, along with the P-value of statistical significance (derived from the F-test under the null hypothesis that all regression coefficients equal zero). If this P-value is smaller than 0.01, we reject the null hypothesis that the line is a constant i.e., that one variable does not differ with the other.

3 RESULTS

We investigate whether the cells' morphology and composition parameters measured by FC (FSC, SSC, and Width channels) are correlated with RNAP levels (FITC channel), and whether these correlations differ with medium richness.

3.1 Growth Rates

We placed cells in LB (control), M63, and TB media. For differences between conditions to be significant, cells should differ significantly in mean

Table 1: Correlation (R^2) between Height (H) and Area (A) for FITC, FSC and SSC in each medium.

	M63		LB			TB	
	R^2	P-value	R^2	P-value	R^2	P-value	
FITCA vs FITCH	0.86	<0.01	0.78	<0.01	0.73	<0.01	
FSCA vs FSCH	0.94	<0.01	0.84	<0.01	0.83	<0.01	
SSCA vs SSCH	0.98	<0.01	0.94	<0.01	0.96	<0.01	

growth rates. This differences were verified in this OD_{600} measurements.

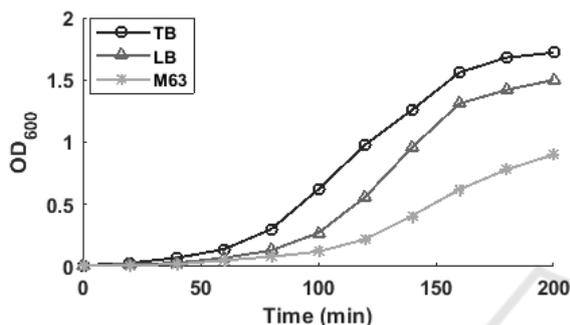


Figure 1: Growth curves of cells of the RL1314 strain in various media, as measured by OD_{600} .

From Figure 1, M63, the poorest medium, has the slowest growth rate, followed by LB and, finally, TB, the richest medium with the fastest growth rate, as expected from previous studies (see e.g. (Goncalves et al, 2018)).

3.2 Correlation between Height (H) and Area (A) of the Flow Cytometer Parameters

Using FC, we extracted the values for FITC, FSC, SSC and W for each cell. The flow cytometer also informs on the ‘Height’ (H) and ‘Area’ (A) of the signals, except for W. We evaluated the correlation between the H and A signals of FITC, FSC, and SSC by least-squares fits (Methods) to measure the R^2 of fitted regression lines, along with the P-value of statistical significance (Table 1).

In all cases we obtained ‘high’ positive R^2 values indicating that the fit approximates well the data, in a positive fashion. Further, all P-values are smaller than 0.01, from which we conclude that the data is well explained by a linear least-squares regression fit between the pairs of variables. As such, from here onwards, we only use the parameters FITCH, FSCH and SSCH, along with W.

3.3 RNA Polymerase Numbers as a Function of Medium Richness

For this, we measured the single-cell fluorescence intensities of RNAP (FITCH channel) in each medium. Figure 2 shows the distribution of the number of cells with given FITCH values for each medium.

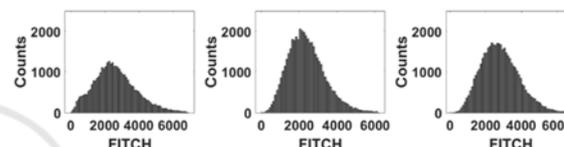


Figure 2: Distribution of the number of cells with given values of FITCH in each medium, as measured by flow cytometry: Left: M63; Middle: LB; Right: TB.

To assess if the distributions differ statistically, we performed Kolmogorov-Smirnov tests (KS-test) of statistical significance between all pairs of conditions (the null hypothesis is that the two data sets belong to the same distribution). In all cases, the P-value was smaller than 0.05, from which we conclude that they differ in a statistical sense.

Table 2: Mean and coefficient of variation (CV) of the distributions of FITCH (proxy for RNAP numbers) in each medium condition.

Medium	Mean(FITCH)	CV(FITCH)
M63	2.6×10^3	0.47
LB	2.4×10^3	0.40
TB	2.8×10^3	0.37

To assess the behavioral trend of RNAP levels with increasing medium richness, we first calculated the mean and coefficient of variation (CV) of each distribution. From Table 2, we find that the CV(FITCH) decreases with medium richness, while the mean(FITCH) is minimized in LB medium.

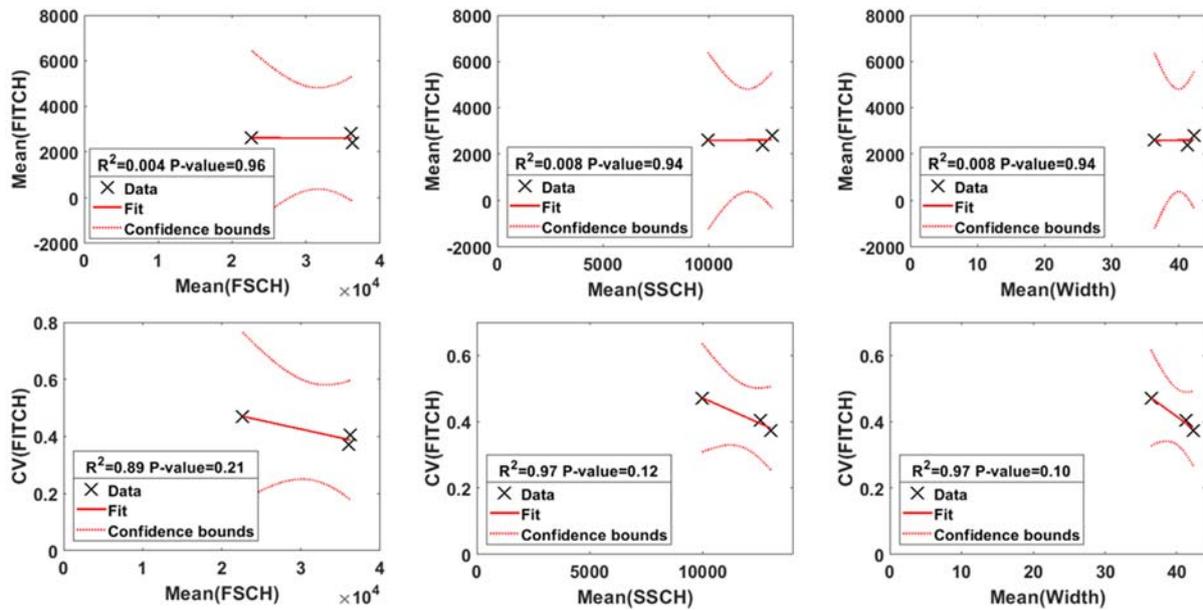


Figure 3: Top: Mean values of FITCH (proxy for RNAP numbers) plotted against the mean values of FSCH, SSCH and Width (proxies for cell size and composition), respectively, in the three media considered (M63, LB, and TB), along with the linear least-squares regression fits and confidence intervals. Bottom: coefficient of variation (CV) of FITCH values plotted against the mean values of FSCH, SSCH and Width, respectively, in the three media considered (M63, LB, and TB), along with the linear least-squares regression fits and confidence intervals.

3.4 Cell Morphology and Composition as a Function of Medium Richness

Next, we investigated how the morphology and composition as seen by parameters obtained by FC differ, at the population level, with medium richness. For this, we obtained the mean and CV of FSCH, SSCH, and W at the single-cell level, in each medium (Table 3). We find that, in general, the mean values of FSCH, SSCH, and W increase with increasing medium richness. Meanwhile, their CV do not exhibit (linear) relationships with medium richness.

3.5 Correlation between Cell Morphology and Composition and RNAP Levels against Medium Richness

To validate the above conclusions, we tested for the occurrence of linear correlations between the mean values of FSCH, SSCH and W with the mean and CV of RNAP levels as a function of medium richness. Figure 3 (Top), shows that there are no such statistically significant correlations.

Similarly, from Figure 3 (Bottom), there are no statistically significant negative correlations between the cell-to-cell variability in RNAP levels and the

mean values of FSCH, SSCH and W. However, if more conditions were considered (e.g. medium of intermediate richness between those tested), linear correlations might become statistically significant.

Thus, we hypothesized that FSCH, SSCH and W, which differ with medium richness, are negatively correlated to the cell-to-cell variability in RNAP, but not to the mean.

3.6 Correlation by Classes between Cell Morphology and Composition and RNAP Levels

From the data, it is visible the presence of much cell-to-cell variability in FSCH, SSCH and W, even within a given medium condition. This hampers the ability to detect correlations between these parameters and RNAP levels.

However, if such correlations exist, they should become enhanced if, instead of analyzing the data based on the growth condition, one instead classifies the cells based on the values of FSCH, SSCH and W (top panels in Figure 4).

Table 3: Mean and coefficient of variation (CV) of FSCH, SSCH, and Width (proxies for cell size and composition) in each medium condition.

	M63		LB		TB	
	Mean	CV	Mean	CV	Mean	CV
FSCH	2.26×10^4	0.27	3.62×10^4	0.21	3.61×10^4	0.15
SSCH	9.98×10^3	0.27	1.26×10^4	0.28	1.30×10^4	0.21
W	36.41	0.12	41.30	0.01	42.29	0.09

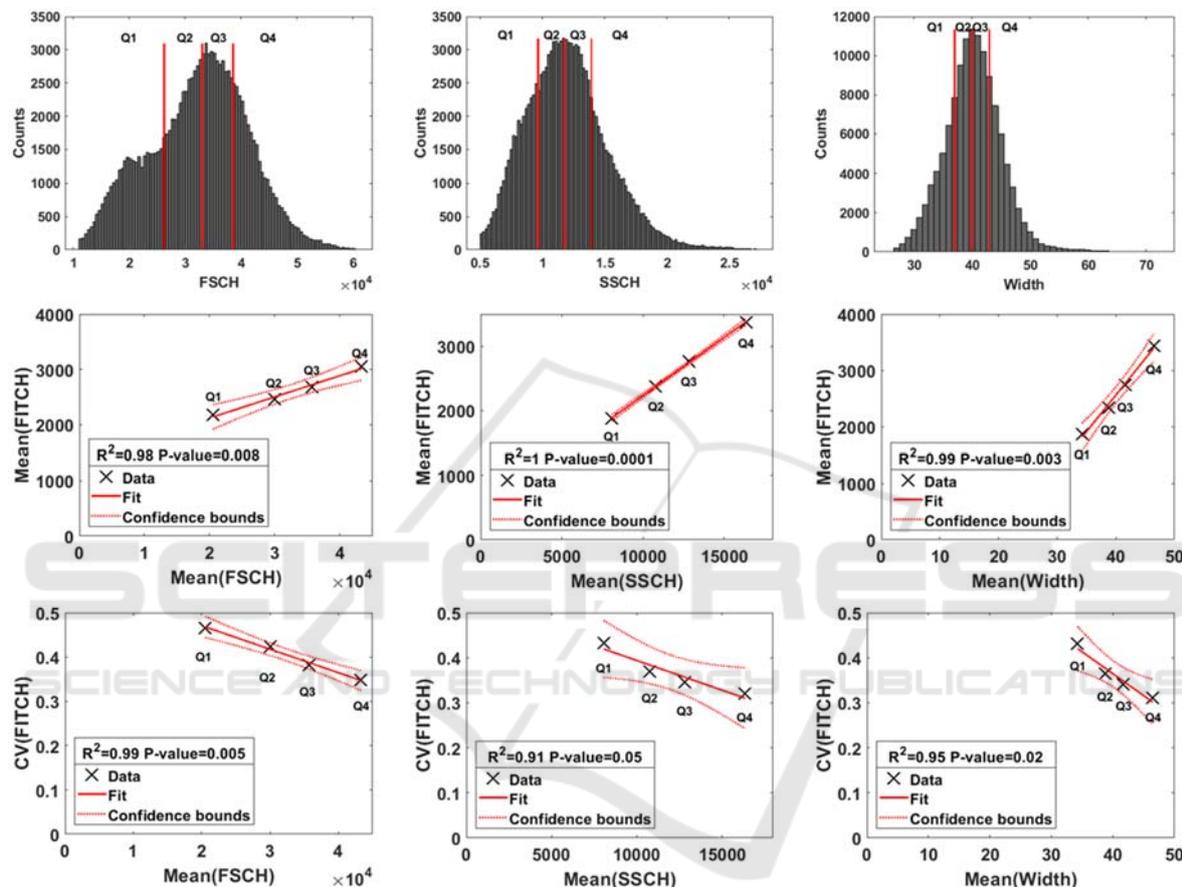


Figure 4: Top: Distributions of FSCH, SSCH and Width (proxies for cell size and composition) values in individual cells from all media; Center: Division of the data sets into quartiles and scatter plots Mean(FSCH) and Mean(FITCH, proxy for RNAp numbers), Mean(SSCH) and Mean(FITCH) , and Mean(Width) and Mean(FITCH); Bottom: Division of the data sets into quartiles and scatter plots Mean(FSCH) and CV(FITCH), Mean(SSCH) and CV(FITCH) , and Mean(Width) and CV(FITCH).

We expect that, if the mean values of SSCH, FSCH, and W can explain the CV(FITCH), then the linear correlations should be equal or stronger than when partitioning the data according to the medium. Further, the P-values should be smaller than 0.01, implying that the correlations are statistically significant.

Figure 4 validates this hypothesis, i.e., when partitioning cells according to the values of SSCH, FSCH, and W, respectively, one finds strong, statistically significant, negative linear correlations.

We conclude that the cell-to-cell variability in RNAp levels decreases for increasing mean values of FSCH, SSCH, and/or W, which are proxies for cell size and/or density. Meanwhile, also from Figure 4, mean RNAp levels increase with mean values of FSCH, SSCH, and W.

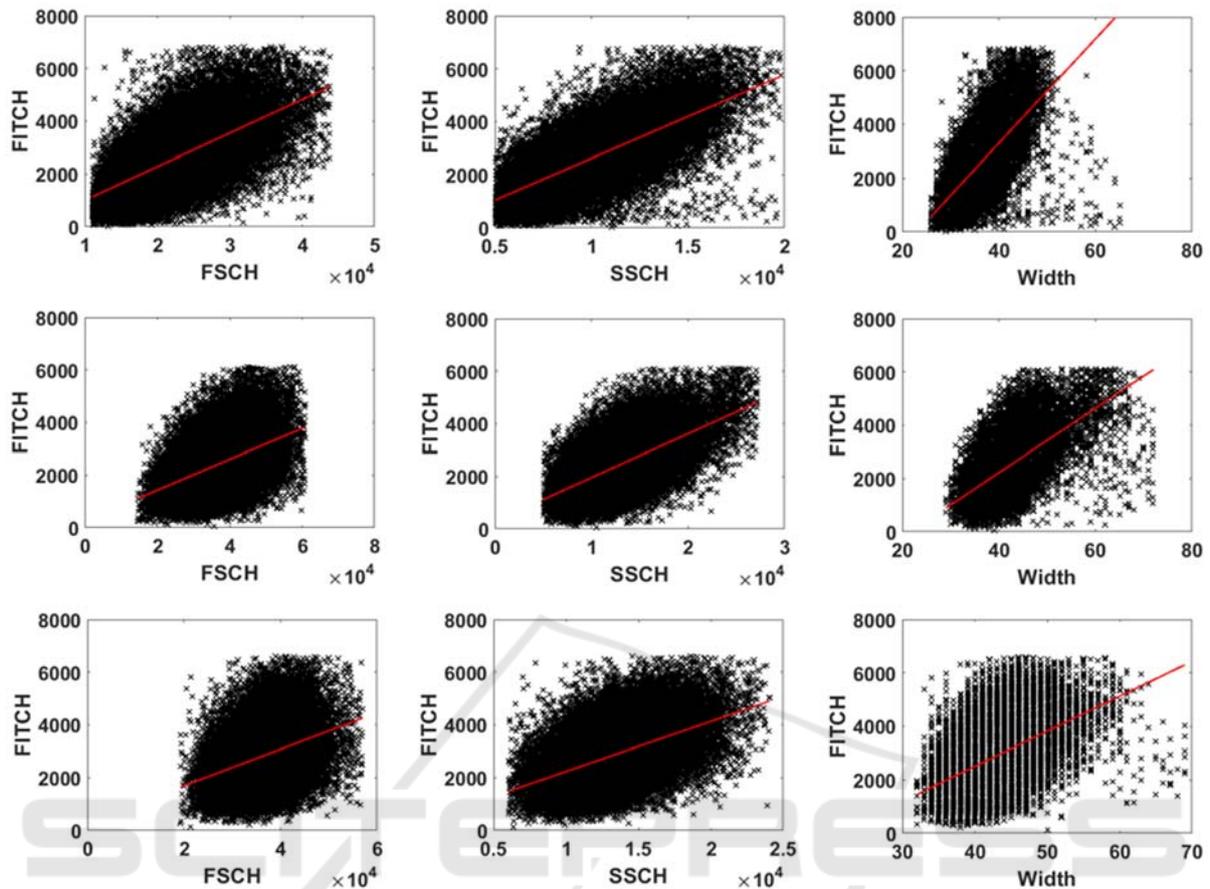


Figure 5: Scatter plots between single-cell values of FITCH (proxy for RNAP numbers) and FSCH, SSCH and Width (proxies for cell size and composition), respectively, in each medium. Top: M63; Center: LB; Bottom: TB. The solid red line is the linear least-squares regression fit.

3.7 Correlation by Classes between the Cell-to-cell Variability in Cell Morphology and Composition and in RNAP Levels

We searched for correlations between the cell-to-cell variability in cell morphology and composition and the mean and the cell-to-cell variability in RNAP levels. To obtain classes of cells with differing variability in these parameters, we made use of random sampling from the entire set of cells gathered from all conditions. Namely, for assembling the values for each class, we randomly selected 10000 cells and obtained the CV of this set. This was performed 1000 times. Next, from the 1000 sets, we selected the 10 sets with minimal and the 10 sets with maximal cell-to-cell variability in FSCH, SSCH, and W, respectively. We obtained the CV of the parameter value for each set, and calculated the average CV of the 10 sets of cells. For each of these sets, we also obtained the mean and

CV of the RNAP levels of individual cells. As we found no statistically significantly linear correlation (R^2 values below 0.15), we conclude that, unlike for mean values, the cell-to-cell variability in SSCH, FSCH, and W, cannot explain the mean and cell-to-cell variability in RNAP numbers.

3.8 Relationship between Cell Morphology and Composition and the RNAP Levels at the Single-Cell Level

Having found a correlation between the mean and cell-to-cell variability in RNAP levels and the mean values of FSCH, SSCH, and/or W of cell populations, we studied whether such correlations are significant at the single-cell level, i.e. in a population of cells in the same medium.

Table 4: Correlation (R^2) between FITCH (proxy for RNAP numbers) and FSCH, SSCH, and Width (proxies for cell size and composition) in each medium.

	M63		LB		TB	
	R^2	P-value	R^2	P-value	R^2	P-value
FITCH vs FSCH	0.39	<0.01	0.21	<0.01	0.13	<0.01
FITCH vs SSCH	0.49	<0.01	0.36	<0.01	0.25	<0.01
FITCH vs W	0.47	<0.01	0.34	<0.01	0.24	<0.01

We searched for correlations between single-cell values of FITCH and the respective values of FSCH, SSCH and W (Figure 5), by performing fits by linear regression (least-squares fit method). Also, we obtained the P-values of statistical significance (Table 4), by applying F-tests (Methods).

From Figure 5 and Table 4, in all media, the linear fits are statistically significant, as the P-values from the least-squares regression fits are smaller than 0.01 (Table 4). Meanwhile, from the R^2 values, we find that the goodness of fit decreases for increasing medium richness.

4 CONCLUSIONS

Our results indicate that the mean and cell-to-cell variability in RNAP numbers in *E. coli* cells differs with parameter values associated to the cell size and composition, as measured by flow cytometry. In particular, the mean increases and the variability decreases as each of these parameter values increases. At the population level, these changes can only be detected by classifying cells according to the values of FSCH, SSCH and Width, respectively. Analyzing the data at the single-cell level, one also finds these correlations, being more pronounced in poor growth medium.

We expect this knowledge to be relevant in studies of gene expression dynamics in various media, as the amount of RNAP is a key regulatory mechanism of transcription dynamics. Namely, our results suggest that the cell-to-cell variability in gene expression may differ not only due to intrinsic noise in gene expression and extrinsic factors, but also due to the medium-dependence of the mean values of FSCH, SSCH and Width.

At present, we cannot explain why the cell-to-cell variability in SSCH, FSCH, and Width are not correlated to the cell-to-cell variability in RNAP numbers, while being correlated to the mean RNAP numbers. An answer to this question may be of relevance, as the RNAP is a master regulator of gene expression in bacteria, and the answer may reveal aspects of how their numbers are regulated. Thus,

the answers should contribute to a better understanding of the modifications that these organisms undergo following environmental changes.

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