Robustness to Sub-optimal Temperatures of the Processes of Tsr Cluster Formation and Positioning in *Escherichia Coli*

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Abstract: Clustering and positioning of chemotaxis-associated proteins are believed to be essential steps for their proper functioning. We investigate the robustness of these processes to sub-optimal temperatures by studying the size and location of clusters of Tsr-Venus proteins in live cells. We find that the degree of clustering of Tsr proteins is maximal under optimal temperature. The data further suggests that the weakening of the clustering process in lower-than and higher-than optimal temperatures is not due to the same cause. Meanwhile, the location of the clusters is found to be weakly temperature independent, within the range tested. We conclude that while the clustering of Tsr is heavily temperature dependent, the localization is only weakly dependent, suggesting that the functionality of the proteins responsible for retaining Tsr-clusters at the cell poles, such as the Tol-Pal complex, is robust to suboptimal temperatures.

1 INTRODUCTION

Escherichia coli have evolved mechanisms to respond to external stimuli, such as chemotaxis. This process is based on clusters of chemoreceptors (Sourjik and Berg, 2004; Wadhams and Armitage, 2004; Parkinson et al., 2005) that can perform multiple tasks, including thermosensing (Lee et al. 1988) and aerotaxis (Rebbapragada et al. 1997).

One identified transmembrane chemotaxis receptor protein is Tsr, a cytoplasmic double membrane-spanning serine receptor (Lee et al., 1988) that preferentially accumulates at the cell poles, where it forms clusters (Thiem et al., 2007).

Clustering and positioning at the poles of the chemotaxis-associated protein clusters are believed to be essential for their proper functioning, namely, they are expected to affect the signal processing capabilities of the receptor system (Kentner and Sourjik, 2006; Vaknin and Berg, 2006; Skidmore et al., 2000; Lybarger and Maddock, 1999), even though changes in receptor activity do not necessarily require changes in the assembly process of the clusters (Liberman et al., 2004).

Recent evidence suggests that the retention of the clusters at the poles is made possible by proteins such as the *trans*-envelope Tol-Pal complex, a widely conserved component of the cell envelope of Gram-negative bacteria (Santos et al., 2014). Little is known about how robust this process to sub-optimal conditions is.

Here, we use the Tsr-Venus construct (Yu et al., 2006) to study, in live *E. coli* cells, the robustness of the clustering process and spatial distributing of Tsr clusters to sub-optimal temperatures. We chose this construct since the tagging of Tsr with Venus does not affect its spatial distribution, due to a linker sequence that allows the cytoplasmic domain of Tsr to freely interact with other signalling proteins, similar to the natural system (Yu et al., 2006). Meanwhile, the Venus protein is a YFP variant, derived from GFP, that has a fast maturation time (Nagai et al., 2002), allowing real time imaging by fluorescence microscopy.

2 MATERIALS AND METHODS

2.1 Chemicals

For routine cultures, the M9 glucose media components, isopropyl β-D-1-thiogalactopyranoside (IPTG), agarose for microscopic slide gel preparation and antibiotics were purchased from Sigma-Aldrich. The amino acids and vitamins were purchased from ThermoFisher.

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2.2 Bacterial Strain and Growth Conditions

We used *E. coli* K-12 strain SX4, harbouring the Tsr-Venus gene construct under the control of the lac promoter (P_{lac}) (Yu et al., 2006), a kind gift from Sunny Xie, Harvard University, U.S.A. Overnight liquid cultures were grown in M9 glucose (0.4%) media, supplemented with amino acids along with the appropriate antibiotics for 14 h, at 37°C with shaking (250 rpm).

2.3 Induction of Tsr-Venus Expression

From overnight cultures, cells were inoculated into a fresh media with the same antibiotics as above, at an initial OD of 0.05 at 600nm, and grown until the OD600 reached ~ 0.3, at 37°C with shaking (250 rpm). The induction of Tsr-Venus expression was performed by adding 200μM IPTG to the culture, which was left in the incubator at appropriate temperature (10°C, 15°C, 24°C, 37°C and 43°C) for 1 hour before imaging.

2.4 Microscopy

To study the formation and localization of Tsr-Venus clusters, 8 μL of cells left at appropriate temperature were then placed on 1% agarose gel pad prepared in M9 glucose media for image acquisition.

Cells were visualized in a Nikon Eclipse (Ti-E, Nikon, Japan) inverted microscope with a C2 confocal laser scanning system using a 100x Apo TIRF (1.49 NA, oil) objective. Images of cells were taken with Nikon NIS-elements. The Tsr-Venus proteins can be detected as fluorescent spots under the fluorescent confocal microscope using a 488 nm argon ion laser (Melles-Griot) and a 515/30 nm detection filter. Images were acquired using a large pinhole, gain 165 and 3.36 μs pixel dwell.

2.5 Image Analysis

Cell segmentation was performed by a custom-made software that integrates MAMLE (Chowdhury et al., 2013) and CellAging (Häkkinen et al., 2013), for cell segmentation. Phase contrast images were automatically segmented and the results were manually corrected as needed. Finally, fluorescence images were automatically aligned to phase-contrast images. The fluorescence in each cell was then extracted and analysed by a custom-made Matlab script.

To assess the location of Tsr-Venus clusters along the major axis of a cell, we first formally defined a 'cluster' or 'spot' as a connected component with each pixel having a light intensity above a threshold. For this, we performed a Laplacian of Gaussian based spot detection combined with an adaptive local thresholding step (Annila, 2015). It assumes that the background pixel intensities follow a Gaussian distribution. The threshold is then selected for each cell separately based on the fitted distribution such that the probability of mislabeling a pixel from this distribution is smaller than 0.005. From the segmented image, the number of clusters was counted and the area of each cluster was calculated by counting the number of pixels within.

To distinguish between midcell and poles, we defined a boundary between them at 0.5 (with 0 being midcell and 1 being the cell extreme) (Santos et al., 2014).

Finally, the intensity of the clusters was directly acquired from background corrected cells, which were obtained by subtracting the median cell intensity from each pixel of the cell. Next, for each cell, the total protein fluorescence was obtained by summing the fluorescent intensity of the clusters.

3 RESULTS

First, after segmenting cells and clusters of Tsr-Venus (see example Figure 1), for each temperature condition, we extracted from each cell the total fluorescence of the clusters, the number of clusters and the area occupied by the clusters (Table 1).

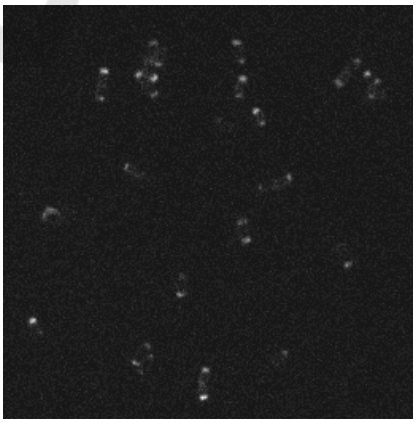

Figure 1: Fluorescence microscopy image of *Escherichia coli* cells expressing Tsr-Venus.

From Table 1, the production of Tsr-Venus proteins under the control of P_{lac} is heavily temperature dependent, as expected (Kandhavelu et al., 2012),

Table 1: Tsr-Venus clustering at different temperatures. Standard error of the mean (SEM) is shown in parenthesis.

	10°C	15°C	24°C	37°C	43°C
No. cells	176	301	259	265	299
Mean total cluster fluorescence (a.u.)	$0.3x10^4$ ($0.02x10^4$)	$0.8x10^4$ ($0.04x10^4$)	$1.5x10^4$ ($0.06x10^4$)	$1.5x10^4$ ($0.08x10^4$)	$0.8x10^4$ ($0.08x10^4$)
Avg. no. clusters per cell	0.53 (0.05)	0.74 (0.03)	1.02 (0.03)	1.23 (0.03)	0.49 (0.04)
Avg. cluster area (μm^2)	0.5 (0.03)	0.74 (0.02)	1.1 (0.03)	1.1 (0.04)	0.8 (0.04)
Clustering coefficient (%)	13.9 (1.4)	30.6 (1.5)	56.2 (1.6)	61.5 (1.2)	18.0 (1.4)

with the total fluorescence per cell from these proteins being maximized at 37°C. Meanwhile, we expect the decrease in mean fluorescence per cell from 37°C to 43°C to likely be due to the increased cell division rate with temperature not being compensated sufficiently by an increase in protein production.

Next, in order to assess whether the clustering process is temperature dependent, we calculated the 'clustering coefficient' of Tsr-Venus. We define this quantity as the ratio between the fluorescence from clusters and the total cell fluorescence. Averages from all cells of these three quantities for each condition are shown in Table 1. Visibly, the clustering coefficient maximizes at 37°C and, thus, we conclude that, under optimal temperature, the Tsr proteins are more efficiently clustered.

We also plotted the mean cluster fluorescence against the mean number of clusters per cell, for each condition (Figure 2). Visibly, there is a lack of linear relationship between temperatures lower than and higher than the optimal. This suggests, as mentioned above, that the underlying causes for weaker clustering at low temperatures differ from the causes at high temperatures. Further studies are needed to identify these causes. We expect division rates and diffusion rates of the clusters to play a role in these differences.

To test whether Tsr-Venus fluorescence is affected by temperature in the range studied, we plotted the mean cluster fluorescence against the mean area of the clusters per cell, for each condition (Figure 3).

From Figure 3, we conclude that bigger clusters tend to be brighter and that this relationship between size and brightness is fairly independent of temperature.

Finally, we studied whether the intracellular localization of the clusters is affected by temperature. For that, we measured the fractions of

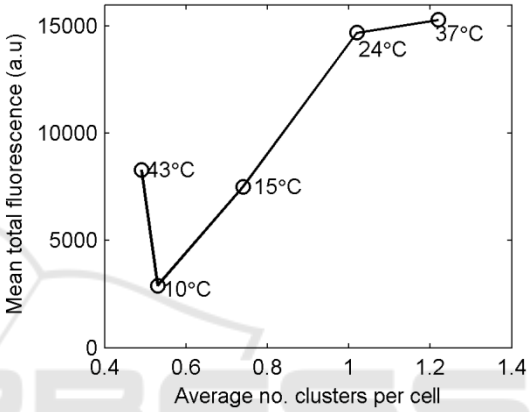

Figure 2: Mean total fluorescence from Tsr clusters as a function of the average number of these clusters per cell.

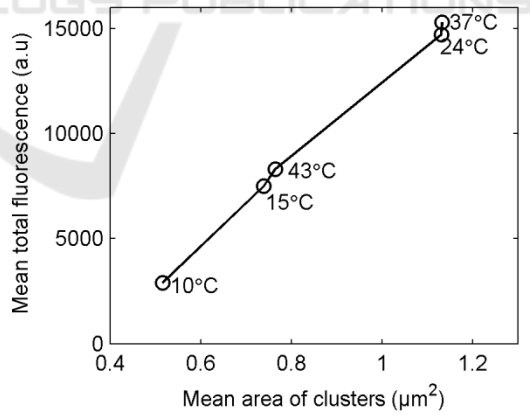

Figure 3: Mean total fluorescence from Tsr-clusters as a function of the average area of the clusters per cell.

'polar' and 'lateral' clusters (Table 2). The former are defined as those inside the poles of the cell, while the latter are at midcell, the region in between the poles.

In all cases, the percentage of polar clusters was over 94%. We performed a binomial test for the proportions of lateral clusters with the null

hypothesis that they are coming from the same distribution. It is usually accepted that, for p-values smaller than 0.01, the null hypothesis is rejected.

Table 2: Localization of Tsr-Venus clusters. Shown are the percentages of clusters at the poles ('polar clusters'), and the percentages of clusters at midcell ('lateral clusters') for cells at 10°C, 15°C, 24°C, 37°C, and 43°C.

	10°C	15°C	24°C	37°C	43°C
Polar clusters (%)	98.9	99.1	99.6	96.0	94.5
Lateral clusters (%)	1.1	0.9	0.4	4.0	5.5

From the comparisons between all pairs of conditions, we find a tangible difference only between 24°C and 43°C (all other p-values were above 0.05). From this, we conclude that the localization process is only weakly dependent of temperature, in the range tested.

4 CONCLUSIONS

Tsr proteins play a central role in the chemotaxis mechanisms of *Escherichia coli*. For this, they participate in large clusters of various proteins.

From microscopy measurements of cells expressing Tsr proteins tagged with Venus proteins, we compared the clustering process and the behaviour of the clusters at different temperatures.

We found that, in all conditions, these proteins are able to form clusters and that these preferentially locate at the cell poles. Nevertheless, the clustering process is temperature dependent in that, at 37 °C, the clusters clearly harness Tsr-Venus proteins more efficiently than in the other conditions.

At the moment, the cause(s) for the dependence of the clustering process on temperature is unknown. However, recent studies showed that both the cytoplasm viscosity (Parry et al., 2014) as well as the relative nucleoid size of these cells are heavily temperature dependent, which could explain why temperature affects the long-term spatial distribution of the clusters. Another possibility is that the clustering process is not energy-free, and thus depends on how much energy the cell has available (similarly to the clustering of unwanted protein aggregates (Govers et al., 2014)).

On the other hand, we observed that the localization of the clusters (at the pole or at midcell), appears to be only weakly temperature dependent, within the range of temperatures studied. From this, we conclude that the functionality of the proteins responsible for retention of Tsr-clusters at the poles, such as the Tol-Pal complex, is robust to suboptimal temperatures, or that other, non-energy dependent mechanisms, contribute to this preference for polar localization.

In the future, it would be of interest to investigate the causes for the temperature dependence of the clustering process and for the temperature independence of the localization process of these proteins.

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