Response to Comment on: "Rivalry in *Bacillus subtilis* colonies: enemy or family?"

Rajorshi Paul[1], Tanushree Ghosh[2], Tian Tang[2], Aloke Kumar[3*]

[1]Department of Mechanical Engineering, Stanford University, Stanford, USA.

[2]Department of Mechanical Engineering, University of Alberta, Edmonton, Canada.

[3]Department of Mechanical Engineering, Indian Institute of Science, Bangalore, India.

***Corresponding author email:** alokekumar@iisc.ac.in

Recently, in a commentary on our work[1], Fernandez et al. highlighted some of their findings which seem to contrast some of our experimental results. Fernandez et al. reported that sibling *Bacillus subtilis* colonies growing adjacent to each other (initial separation 10 mm) in salt media formed a demarcation zone initially which eventually was resolved by colony "abuttal". Their confocal microscopy of colonies showed that colonies which did not form a demarcation line do not mix over time. Colonies separated by large distances were still found to exhibit asymmetry and form zones of demarcation, in line with our findings. Further, Fernandez et al. suggested that the mathematical model proposed by us does not account for transport of biochemical inhibitors which was implicated in inter-colony interactions in our paper. While we appreciate their efforts in examining and commenting on our work, we would like to clarify on a few issues in this response. All experiments reported here were performed in growth medium prepared by mixing 15 g L^{-1} Miller's Luria Broth (LB) base with bacteriological agar. The composition of LB and agar is the same as reported in our paper[1].

In our study, the term demarcation line (DL) is used to refer to the observation where a region between two colonies devoid of bacteria can be observed. The phenomenon of demarcation in colonies has been well documented[2-11]. On the other hand, when no such region exists and the two colonies meet, the colonies are said to have merged. This qualitative observation can be quantified either macroscopically or microscopically.

Macroscopically, the distinction between demarcation and merging could be made by analyzing the optical intensities across the colonies. If colonies form a DL, then the intensity over the demarcation region would be similar to that of the background. In the case where a DL line exists, a sharp change of optical intensities can be expected to occur. This is demonstrated in Fig. 1a and 1b, which contrasts optical intensities in a case of no DL formation (Fig. 1a) vis-à-vis a DL formation (Fig. 1b). The insets show the gray scale image of colonies collected from the experiments. To eliminate the effect of uneven background illumination, the gray values in Fig. 1a and 1b were obtained from the blue channel of raw images using ImageJ. The raw images from the experiments correspond to 48 hours post inoculation. Although we do not have access to raw data of Fernandez et al., it is likely that the colonies shown in Fig. 1a and 1b of the commentary by Fernandez et al. will be considered to have merged, while the interaction between four colonies as shown in Figure 1d of the commentary does form a region of demarcation. To shed more light on the dynamics at the line where the colonies meet, the colonies were imaged with an optical microscope (Fig. 1c and 1d). A crease is observed at the line of intersection where the two colonies meet (Fig. 1c). The crease folds as the colonies overlap, which is the dark zone in Fig. 1d. The images in Fig. 1c and 1d correspond to the colonies at the same instant of time after they have merged but obtained at different locations along the line of intersection. It seems that the event of merging at the microscale is a rich and open question. We have focused on the inter-colony

interactions at macroscopic length scale and microscopic detail in the merging phenomenon is out of scope in our study.

In the commentary, Fernandez et al. explore the dynamics of inter-colony interactions in a salt medium which is distinctly different from the composition of our growth medium. It is well known that composition of the growth medium and the stiffness of the substrates influence bacterial growth and propagation[7]. Salt media is known to influence the rate of spreading of colonies and can stifle colony growth. Further, the authors used a strain of *Bacillus subtilis* which is different from the one used by us. By studying sibling interactions in two closely related strains of *Paenibacillus dendritiformis*, Be'er et al. showed that inter-colony interaction depends of the strain of bacteria used[9]. Therefore, the findings reported by Fernandez et al. sheds new light on the inter-colony dynamics, but their results are not necessarily in conflict with those reported by us.

Our experimental results correspond to bacteria colonies incubated for 60 hours at 37°C. Longer incubation was not reported as mutations in the cells over time can alter the behaviour of the cells and result in unwarranted artifacts. However, colonies which were incubated for 116 hours post inoculation do show that the demarcation line persists, and colonies do not merge even if they are incubated for long periods (Fig. 1e). The persistence of the DL seems to be a trend with colonies which do not merge, e.g., Fig. 7 of our article[1] shows the persistence of demarcation lines in *P. fluorescens* and *E. coli* incubated for a week.

Our mathematical model is phenomenological and the parameters defined in the model are based on experimental observations. One of the implicit assumptions in the model is that the formation of demarcation lines is due to transport of some biochemical inhibitors. A detailed examination of the molecular pathways responsible for inhibition and consequent identification of relevant proteins is out of scope for the present study. Therefore, the effect of the biomolecular inhibition

was lumped into the mortality rate *m(t)* defined in the article. The reference time *t** was also introduced to account for the time scale of the biomolecular diffusion. An explicit transport equation for the inhibition factors was not formulated. We have recognized this limitation of the model and the point has been discussed in detail in the paper (final paragraph of section 4[1]).

The dynamics of inter-colony interactions in bacteria present an intriguing open problem and much of it remains unexplored. The resilience and robust adaptation of bacteria makes this problem an interesting one full of possibilities. New findings by Fernandez et al. attest to the diversity that colony interactions in bacteria can exhibit. In our opinion, the study by Fernandez et al. differs from our investigation[1] and the results from the two studies are not in conflict but reveal two different aspects of inter-colony interactions. Preservation of identity of merged colonies is an exciting new development and may hold key to answering interesting questions on inter-colony cooperation post merging.

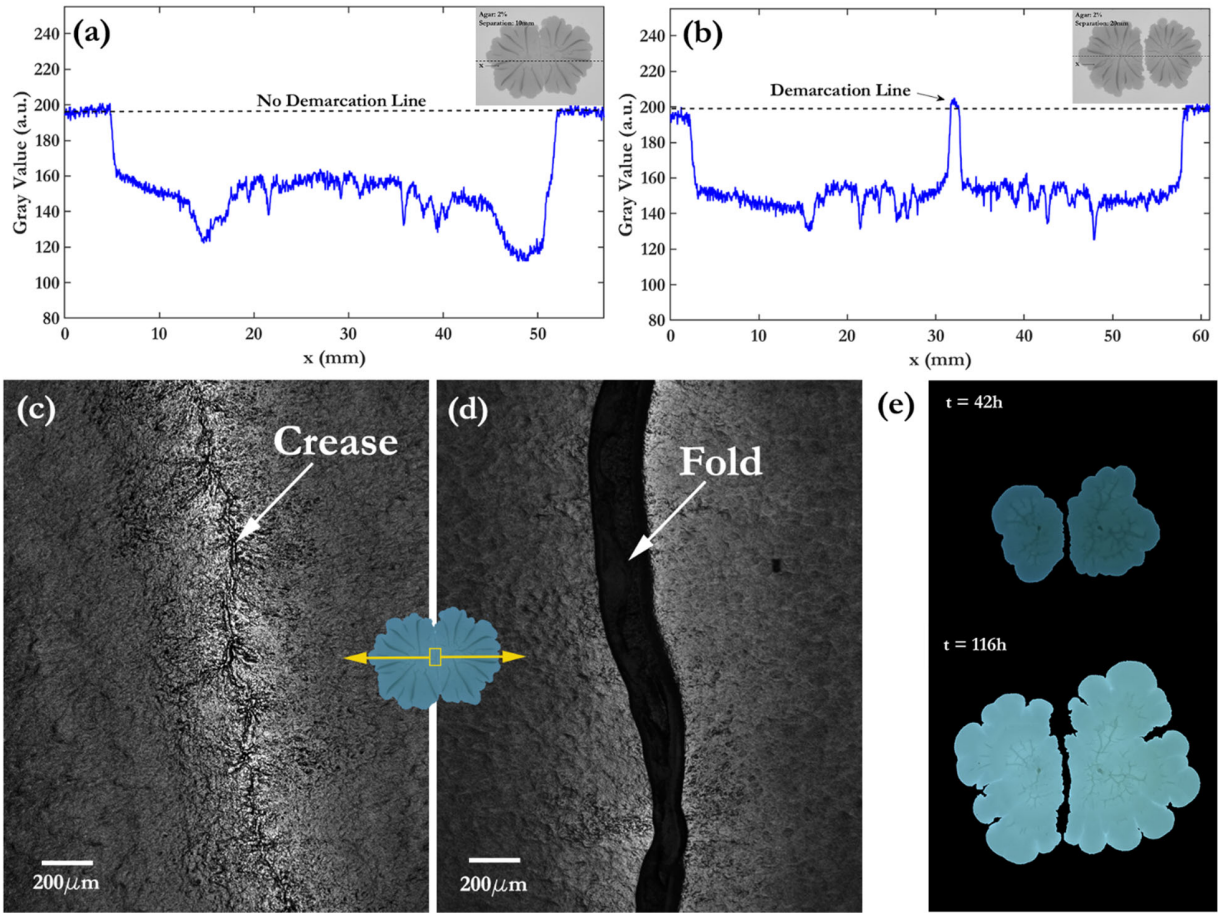

Fig. 1. (a - b) Quantitative distinction between **(a)** merging and **(b)** DL formation. The pixel intensity at the DL matches the background intensity (denoted by the dotted black line), while for merged colony this does not occur. The insets show the gray scale image of the colonies. The pixel intensities were measured over the dotted lines. **(c - d)** The line of intersection between two merged colonies growing on 2% agar, 10 mm apart, observed under a confocal microscope. **(c)** A crease forms at the line of intersection. **(d)** The folding of the crease occurs over time denoted by the dark zone (right). **(e)** The DL formed between colonies growing on 2% agar with separation of 15 mm persists at 116 hours after inoculation.

References

1. R. Paul, T. Ghosh, T. Tang and A. Kumar, *Soft Matter*, 2019, **15**, 5400-5411.
2. L. Dienes, *J Bacteriol*, 1946, **51**, 585.

3. L. Dienes, *Proceedings of the Society for Experimental Biology and Medicine*, 1947, **66**, 97-98.
4. B. W. Senior, *J Gen Microbiol*, 1977, **102**, 235-244.
5. A. Be'er, H. P. Zhang, E. L. Florin, S. M. Payne, E. Ben-Jacob and H. L. Swinney, *P Natl Acad Sci USA*, 2009, **106**, 428-433.
6. A. E. Budding, C. J. Ingham, W. Bitter, C. M. Vandenbroucke-Grauls and P. M. Schneeberger, *J Bacteriol*, 2009, **191**, 3892-3900.
7. A. Sekowska, J.-B. Masson, A. Celani, A. Danchin and M. Vergassola, *Biophysical journal*, 2009, **97**, 688-698.
8. A. Be'er, G. Ariel, O. Kalisman, Y. Helman, A. Sirota-Madi, H. P. Zhang, E. L. Florin, S. M. Payne, E. Ben-Jacob and H. L. Swinney, *P Natl Acad Sci USA*, 2010, **107**, 6258-6263.
9. A. Be'er, S. Benisty, G. Ariel and E. Ben-Jacob, in *The Physical Basis of Bacterial Quorum Communication*, ed. S. J. Hagen, Springer New York, New York, NY, 2015, DOI: 10.1007/978-1-4939-1402-9_8, pp. 145-162.
10. B. Chakraborty, A. Mallick, S. Annagiri, S. Sengupta and T. K. Sengupta, *R Soc Open Sci*, 2016, **3**, 160438.
11. P. Patra, C. N. Vassallo, D. Wall and O. A. Igoshin, *Biophysical Journal*, 2017, **113**, 2477-2486.