nature portfolio

Peer Review File

Collective condensation and auto-aggregation of Escherichia coli in uniform acidic environments

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

Collective condensation and auto-aggregation of bacteria in uniform acidic environments.
Nir Livne, Moriah Koler and Ady Vaknin*

This article was very comprehensive in regard to its technicality.
The scientific strategy was very well thought-out.
The outcome of this scientific endeavor deserves publication.

This article builds on a recent publication from Livne and Vaknin.
Collective responses of bacteria to a local source of conflicting effectors. Nir Livne & Ady Vaknin. Scientific Reports volume 12, Article number: 4928 (2022)

I have composed a small list of potential improvements.
Many of the items in the list correspond to the fact that the manuscript is written for a much more knowledgeable audience. The authors feel very comfortable using terms like "planktonic" "aggregates" "condensates" and "biofilm" without explaining the terms or their similarities or differences.
The manuscript is highly specific and there is no discussion of applications to human health or extrapolations to other aspects of microbiology.

1 Collective condensation and auto-aggregation of bacteria in uniform acidic

2 environments.

Is this appropriate? UNIFORM (opposing local changes) and ACIDIC ENVIRONMENTS (organic)?

24 Abstract is long and includes unexplained items Ag43

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It is not clear how the methods described previously are “growth-related”

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154 the experiments shown in Fig. 1C were repeated using cells lacking either the Tsr, Tar, Aer, or

155 both Tar and Aer chemoreceptors.

There is no explanation, so far, of the function of these proteins or their relationship to each other, if any.

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265 "non-aggregating cells" are these the same cells? How are they different than the previously used E coli, MG1655, etc.?

Figure 3 Introduces the concept that the protein systems correspond to the inner membrane. This is relevant since later "nonboring" is also used (line 150). Also, OD is missing the wavelength. The wavelength is missing in all figures.

Confirm that this line is written as intended.

334 Additionally, by fostering cell aggregation and potentially biofilm formation, cell condensation At this point in the manuscript, or even before, it will be very beneficial to explain in plain terms the differences or similarities between biofilm, cell aggregation, planktonic cells, Ag43 knockout cells, etc. How is this related to Quorum Sensing? Is this aggregation a phenomenon of the bacteria in the wild? Where are these levels of pH encountered by bacteria? Human environments are regulated to maintain pH ranges between 7.32-7.42. Perhaps inside the kidney where there are some acidic environments close to 5 or 6. Can bacteria colonize tumors? One of the properties of solid tumors is their acidity on the extracellular leaflet of the plasma membrane.

353 OD600 0.4. Notably, this medium supports long-term bacterial motility but not growth. This is an important factor that should have been part of the main text and not only the Materials and

Methods.

Section 1 – The model and its numerical solutions. The model used through our calculations includes the following equations

In spite of the extensive explanations in the SI, I am left to consider MODEL of what exactly? Aggregation? Bacterial Condensation?

Reviewer #2 (Remarks to the Author):

It was an absolute pleasure to read the work of Livne, Koler and Vaknin. There is very little, if at all, to dislike about this paper.

In my view, this paper contains two discoveries. First, the authors discovered a novel mechanism of bacterial condensation, which is driven by a collective 'inward' movement of bacteria that run down a chemorepellent gradient, generated by the absorption of chemorepellent by the population. This discovery comes at a backdrop of increased attention for collective migration patterns (mostly outward, but also inward) that are driven by self-generated chemoattractant gradients. To my knowledge, this is the first observation of collective behavior driven by chemorepulsion. Second, they discover that under certain experimental conditions (depending on temperature), the bacterial condensates not only contain swimming cells but also aggregates of cells, that are expelled to the periphery by the collective pushes of the motile cells in the condensate center. Both phenomena are very interesting to a broad audience (biologists in general, but also soft matter physicists, and modellers interested in emerging phenomena). Although presented together and obviously connected, each phenomenon easily deserves its own paper, making this manuscript very rich and very likely to generate follow-up studies. I highly recommend it for publication.

Scientifically, I have only one main comment:

-Can the authors completely rule out the role of the secretion of quorum sensing molecules (like AI-2) in the center of the condensate? The authors rightfully say that the timescale and shape of aggregates formed by only AI-2 are different (e.g. as reported in Ref 34), but this does not exclude the possibility that in addition to self-generated chemorepellent gradients there is an additional (even minor) role of secreted chemoattractant in the middle of the condensates (especially because Tsr is so important for these condensates). The authors report one control experiment to address this (Fig S5), which shows no chemoeffectors are produced by the cells, but one could argue that this evidence is not very direct as the spatial structure of the condensates is lost or averaged out. I think the evidence from the modelling is sufficient to show that the phenomenon is in principle explainable without any chemoattractant, but it would be good to comment on this possibility explicitly, for example in the discussion.

The rest of my comments are mostly with respect to improving the clarity of the manuscript and are optional.

1. As said the paper is very rich and could benefit from some streamlining. There are several ways to do that, I give here one concrete example. I would combine fig 1B and Fig 2 and place this in the supplement. I suspect the authors discovered the phenomenon by the experiments in as reported in Ref 19 (Fig 1B) but for an uninformed reader this way in is not required. The authors can simply say they wanted to investigate the response of cell suspensions to pH stress when connected to a larger basin, present their discovery and then say this finding is robust in other experimental environments.
2. The model should be included in the results section and the discussion can devote some more attention to the functional benefit that these collectives bring, and potentially other ways to connect it to the physiology and ecology of bacteria. The phenomenon seems robust enough to be directly encountered in nature, and it is expected to have a functional benefit as providing pH stress protection. This is stated as a mere fact (L309) but is important enough for explicit discussion and the trade-offs involved (e.g. more on the trade-off with oxygen), and perhaps also the reversibility of this process. Overall, comparing it to other pH stress response techniques are helpful. Many follow up experiments are possible to deepen the ecology/physiological side of the story, beyond the scope of this paper, but the authors could talk about this more and set the stage for future work.
3. “Chemotaxis is inherently single-cell” L25 (and L65). I know what the authors mean, but I disagree with this statement. Yes, the signal transduction happens in a single cell. But the network architecture can be shaped by selection acting on the collective motion. The lab of Thierry Emonet has produced some interesting papers on this. Concrete example: I think the very strong preference of E coli for serine and aspartate is not only determined by their metabolic values, but could be also be the result on selection of collective migration patterns driven by serine/aspartate uptake and self-generated gradient generation. It’s also not necessary for any of the claims made by the authors that bacterial chemotaxis is inherently single-cell.

And then some minor issues:

L100. It was my understanding that motility buffer does not support growth for E coli RP437 because of an auxotrophic condition in that strain. It could be that it also does not support growth

for MG1655, which is not an auxotroph, but in that case a reference would be needed.

L78. The embedded population is referenced before the isolated population is discussed. So part of Fig 1A is not really used.

Fig 4B add explicitly that increase in intensity is increase in pH

L243 Temperature comes in quite abruptly. Can the authors add a rationale or hypothesis of why they explored this parameter?

L282: weird formatting: "(Fig. 1-2), illustrated in Fig. 7A."

Fig 1C it is my understanding that the complete area outside of the area indicated with a blue rectangle is hydrogel. Perhaps indicate this explicitly in the figure (e.g. make it darker for example).

I think it would be good to remind the reader explicitly about the differences in scale between AI-2 induced auto-aggregation and condensation. It may be required for a biology audience to more explicitly introduce and distinguish these two concepts as I anticipate for some readers this distinction is somewhat blurry.

I find the phrasing "The experiments done in Fig 1C" not very elegant. Just give the experiment a name and refer to it in the text.

Addendum regarding the modelling part of the study:

Although the main contribution of this work is experimental, the numerical model adds quantitative insights, and hence also deserves more attention in the main text. The modelling aspect of the study seems overall carefully done but I have two sets of comments and suggestions:
-Section 1 seems in principle in line with previous descriptions of population-movement models. It would be very useful if the authors add the relevant references here (e.g. to Keller-Segel and important applications). However, the uptake term of protons is different from the typical uptake term that is used for many chemotaxis models. The shape of the term (linear) seems well constrained by data (Fig S7), but I had expected a Michaelis-Menten term here. The reason is that cells cannot simply absorb an arbitrary amount of ions (could the authors calculate how many ions each cell has to take up in this case?), and likely they need to use an enzymatic reaction to neutralize the protons after absorption. I expected this reaction at some point to saturate for a given amount of cells. Could the authors explain why this curve remains linear?
-The entire method section in the supplement could benefit from editing, to bring it to the same level as the main text in terms of clarity. It should be at a level that another researcher (say another biophysicist) could reproduce the steps. Some examples: Section 1 has an unlabeled figure in the middle of the text. Section 2 and 3 are set up rather minimalistically and I think the authors ought to add more explanations and references here. The origin of the unnumbered equation in section 2 comes out of the blue (I believe it is just a phenomenological description). Also a sentence like "Eq. 2 was then used to estimate the chemotactic drift velocity" is too minimalistic. In Section 4, the

parameter \alpha is not defined. Can the authors also provide an intuitive explanation what the parameter \epsilon means?

We appreciate the reviewers' efforts and the time spent in carefully reviewing the MS. Their suggestions clearly have improved the MS both scientifically and in terms of its clarity.

Reviewer #1

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Nir Livne, Moriah Koler and Ady Vaknin*

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The scientific strategy was very well thought-out.
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The manuscript is highly specific and there is no discussion of applications to human health or extrapolations to other aspects of microbiology.

We very much appreciate the reviewer feedback, which helped us to clarify the MS.

1 Collective condensation and auto-aggregation of bacteria in uniform acidic environments. Is this appropriate? UNIFORM (opposing local changes) and ACIDIC ENVIRONMENTS (organic)?

We are not sure what the reviewer meant here. Indeed, condensation relies on the bacteria collectively modulating the environment, which, in turn, leads to their condensation. These collective responses are initiated in an initially uniform (low pH) environment, with no gradients.

24 Abstract is long and includes unexplained items Ag43

The abstract has been shortened and the term 'Ag43' removed.

E coli was not introduced to the reader. Is this organism used because of its easy access? Does it mimmic other species of bacteria with clinical applications?

E. coli is a well-known model organism for studying bacterial chemotaxis. It is now introduced on lines 52-54.

43 The ability of bacteria to tolerate a pH... All bacteria have the same ability, the same challenges? Gram positive, Gram negative? Acidophillus, etc...

Different bacterial species indeed differ in their preferred pH. Yet, the mere existence of such optimal pH is quite generic. Substantial deviation from this optimum clearly leads to growth defect. Ref. 2, was added in the first line of the introduction to cover also gram-positive bacteria, and refs. 51-52 were added and discussed in lines 358-361, mentioning more generic behaviors in other bacteria (361-365).

44 In particular, organic weak acids, which ... Name a few, to make the story enter focus.

We revised line 40 to include lactate and acetate.

50 under conditions where these growth-dependent mechanisms are ineffective. It is not clear how the methods described previously are "growth-related"

Indeed, this was not defined clearly enough. The chemotaxis system is expressed under a wide range of conditions, not specific to pH stress. Thus, when facing an acid stress, it can promptly respond by swimming away from the low pH region. In contrast, the expression of acid resistance (AR) components is differentially expressed when cells face low pH. These adaptive mechanisms often involve cells growing in the presence of specific amino acids (see refs. 1 and 15). We suggest here that these adaptive mechanisms can operate more efficiently if the acid stress is first partially mitigated by spontaneous chemotaxis-mediated cell condensation.

To clarify, we removed now the reference to growth from the first paragraph, and refer to these pH-specific systems in lines 89-91 and 347-349.

60-64 Please explain the difference between migrating or dwelling. The lack of these protein systems present danger to the life or survival of E coli (other bacteria)?

As indicated, the chemotaxis receptors are essential for chemotic responses to variety of environmental ques, including pH, and thereby controls the distribution of bacteria in the environment, (see references therein). Thus, these proteins do not directly involve in countering the pH stress itself (as other AR systems), except trying to avoid the acid environment altogether by swimming away from the acidic regions. We now explicitly mention this in lines 50-51.

77-90 This is out of place. No citations are included. It looks like a summary of their work. Thus, belongs in the discussion or conclusions.

According to the Comms. Biol.'s guide, the last paragraph of the introduction should include a brief summary of the main results and conclusions.

93 Should introduce the type of E coli (MG1655 cells) and their properties that makes them useful for the study

We now clarify this in lines 52-54 and references therein. Notably, MG1655 is a commonly used strain for studying chemotactic behavior. For example, see also:

Yang, Y., M. Pollard, A., Höfler, C., Poschet, G., Wirtz, M., Hell, R., & Sourjik, V. (2015). Relation between chemotaxis and consumption of amino acids in bacteria. Molecular microbiology, 96(6), 1272-1282.

Partridge, J. D., Nhu, N. T., Dufour, Y. S., & Harshey, R. M. (2019). Escherichia coli remodels the chemotaxis pathway for swarming. MBio, 10(2), 10-1128.

107 chemical coupling Please explain what is meant by this term? In line 235 the term 'coupled' is used, not sure is meant for the same terms.

'Chemical coupling' is used to indicate efficient exchange of chemicals through diffusion between the two environments. This is now clarified in lines 101-104 and 108-112.

154 the experiments shown in Fig. 1C were repeated using cells lacking either the Tsr, Tar, Aer, or
155 both Tar and Aer chemoreceptors.
There is no explanation, so far, of the function of these proteins or their relationship to each other, if any.
Tar and Tsr were mentioned in the introduction, and Aer, indeed, was mentioned only later. We now reiterate the roles of Tar and Tsr, as well as Aer, at the first paragraph of this section (lines 145-148).

252 condensed planktonic cells, what is meant for this term?

'Planktonic bacteria' usually refer to free living (swimming/suspended) bacteria, contrasted with bacteria that exist in aggregates or dense communities, known as biofilms. We now clarify the term in lines 87 (and 285).

265 "non-aggregating cells" are these the same cells? How are they different than the previously used E coli, MG1655, etc.?

These are the same (MG1655) cells that were pre-grown at 33.5C instead of 37C. Note that cells pre-grown at 33.5C did not exhibit aggregation, most likely due to reduced expression of Ag43. We now state this finding more clearly in lines 276-281 and included a new Fig. S9.

Figure 3 Introduces the concept that the protein systems correspond to the inner membrane. This is relevant since later "nonboring" is also used (line 150). Also, OD is missing the wavelength. The wavelength is missing in all figures.
Confirm that this line is written as intended.

We apologize for the confusing typo: "nonboring" should be 'neighboring'. This section was anyway revised in response to other comments. We now added the wavelength (600 nm), in either the axes titles or legend.

334 Additionally, by fostering cell aggregation and potentially biofilm formation, cell condensation At this point in the manuscript, or even before, it will be very beneficial to explain in plain terms the differences or similarities between biofilm, cell aggregation, planktonic cells, Ag43 knockout cells, etc.

Indeed, since the term 'cell aggregates' sometimes refer to different behaviors, we now, upfront, introduce the distinction used here between 'cell condensation' and 'cell aggregation', and their distinct spatial scales (lines 73-75). This distinction is also explicitly shown in Fig. 6a and highlighted in Fig. 6b. We also include a brief explanation of the role of Ag43 in line 294; explain the term 'planktonic cells' in line 87, and 'biofilm' in lines 88-89.

How is this related to Quorum Sensing? Is this aggregation a phenomenon of the bacteria in the wild? Where are these levels of pH encountered by bacteria? Human environments are regulated to maintain pH ranges between 7.32-7.42. Perhaps inside the kidney where there are some acidic environments close to 5 or 6. Can bacteria colonize tumors? One of the properties of solid tumors is their acidity on the extracellular leaflet of the plasma membrane.

The bacterial condensation revealed here does not directly rely on quorum sensing or AI-2 signaling. However, once condensation occurred, it can indeed be affected by quorum sensing and AI-2 chemotaxis. We now discuss the potential role of AI-2 chemotaxis in lines 365-367 and 372-375.

Bacterial aggregation and biofilms are clearly observed in nature, see for example refs. 43 and 54.

As mentioned in the first paragraph of the introduction (lines 39-43), low pH environments are common in nature, including the stomach, gut, urinary tracts, as well as in plants and food products. We appreciate the reviewer suggestion to look into low pH in tumors. We now added this to line 43 and references therein.

353 OD600 0.4. Notably, this medium supports long-term bacterial motility but not growth. This is an important factor that should have been part of the main text and not only the Materials and Methods. We now emphasize this in the main text (line 96).

Section 1 – The model and its numerical solutions. The model used through our calculations includes the following equations
In spite of the extensive explanations in the SI, I am left to consider MODEL of what exactly? Aggregation? Bacterial Condensation?

The model accounts for the bacterial condensation (not aggregation). As suggested by reviewer 2, we now briefly introduce the model in the text. To clarify the model's purpose, we explicitly mentioned it now in lines 202-204.

Reviewer #2

It was an absolute pleasure to read the work of Livne, Koler and Vaknin. There is very little, if at all, to dislike about this paper.

In my view, this paper contains two discoveries. First, the authors discovered a novel mechanism of bacterial condensation, which is driven by a collective 'inward' movement of bacteria that run down a chemorepellent gradient, generated by the absorption of chemorepellent by the population. This discovery comes at a backdrop of increased attention for collective migration patterns (mostly outward, but also inward) that are driven by self-generated chemoattractant gradients. To my knowledge, this is the first observation of collective behavior driven by chemorepulsion. Second, they discover that under certain experimental conditions (depending on temperature), the bacterial condensates not only contain swimming cells but also aggregates of cells, that are expelled to the periphery by the collective pushes of the motile cells in the condensate center. Both phenomena are very interesting to a broad audience (biologists in general, but also soft matter physicists, and modellers interested in emerging phenomena). Although presented together and obviously connected, each phenomenon easily deserves its own paper, making this manuscript very rich and very likely to generate follow-up studies. I highly recommend it for publication.

We very much appreciate the reviewer opinion. We indeed considered splitting the MS into two papers. However, we eventually thought that the inherent natural connection between the parts might come across better as one story.

Scientifically, I have only one main comment:

-Can the authors completely rule out the role of the secretion of quorum sensing molecules (like AI-2) in the center of the condensate? The authors rightfully say that the timescale and shape of aggregates formed by only AI-2 are different (e.g. as reported in Ref 34), but this does not exclude the possibility that in addition to self-generated chemorepellent gradients there is an additional (even minor) role of secreted chemoattractant in the middle of the condensates (especially because Tsr is so important for these condensates). The authors report one control experiment to address this (Fig S5), which shows no chemoeffectors are produced by the cells, but one could argue that this evidence is not very direct as the spatial structure of the condensates is lost or averaged out. I think the evidence from the modelling is sufficient to show that the phenomenon is in principle explainable without any chemoattractant, but it would be good to comment on this possibility explicitly, for example in the discussion.

As the reviewer pointed out, chemotaxis to AI-2 is also mediated by Tsr, and so the importance of the Tsr receptors to cell condensation could indeed suggest that self-induced AI-2 taxis may contribute to the observed bacterial condensation. However, a line of evidence indicated that the primary driving force of the condensation is pH taxis. These include the Tar-dependent impediment of the condensation, the need for a low pH environment, and, importantly, the fact that addition of strong buffer, even at low pH, diminished the condensation. Nevertheless, prompted by the reviewer comment, we further tested now whether bacterial condensation inherently relies on Tsr. To this end, we conducted similar experiments, as in Fig. 1c, except that the pH was uniformly set to alkaline pH (9.6), and lactate was replaced with glycerol as the energy source. The rationale was as follows: under such conditions the cell population tends to lower the pH, rather than increasing it, and therefore a reversed pH gradient can be expected, i.e., lower pH where the cell density is elevated and high pH at the periphery. As shown now in Fig. 3e, bacterial condensation was evident also under these (reversed)

conditions, with the roles of Tar and Tsr indeed reversed; in this case, Tar was essential for condensation rather than Tsr. This is discussed in a new section titled 'Condensation in alkaline pH: the roles of Tar and Tsr' (line 188).

Yet, it is still possible that once condensed, other factors, like quorum-sensing and AI-2 taxis may affect the shape of mature condensates. We discuss this now in lines 365-367 and refer to ref. 32 there.

Finally, local secretion of AI-2 may also affect the bacterial aggregation (rather than condensation), see ref. 40. Interestingly, as in AI-2 mediated aggregation, local fluctuations of the pH, on the scale of the inter-bacterial distance, may also contribute directly to aggregation, not only by inducing large-scale condensation. We now discuss these possibilities in lines 372-375.

The rest of my comments are mostly with respect to improving the clarity of the manuscript and are optional.

1. As said the paper is very rich and could benefit from some streamlining. There are several ways to do that, I give here one concrete example. I would combine fig 1B and Fig 2 and place this in the supplement. I suspect the authors discovered the phenomenon by the experiments in as reported in Ref 19 (Fig 1B) but for an uninformed reader this way in is not required. The authors can simply say they wanted to investigate the response of cell suspensions to pH stress when connected to a larger basin, present their discovery and then say this finding is robust in other experimental environments.

Indeed, introducing the 'channel' assay at the beginning (previous Fig. 1B) is not necessary. However, underlying this choice was also the intention to emphasize that 'embedded' populations can be realized not only in static physical constrains, but also dynamically, by other environmental cues.

Thus, we partly excepted the reviewer suggestion by removing Fig. 2, but also making the following changes:

Fig. 2a was moved to the SI (as suggested).

Fig. 2b (the agarose-plate experiments) moved to Fig. 1b. We believe that this assay is simpler, more intuitive, and more directly related to the experiments in Fig. 1c. Thus, it was introduced first.

Fig. 1b (the channel assay) is now shown in Fig. 5, emphasizing the dynamic separation of the bacteria from the larger environment.

2. The model should be included in the results section and the discussion can devote some more attention to the functional benefit that these collectives bring, and potentially other ways to connect it to the physiology and ecology of bacteria. The phenomenon seems robust enough to be directly encountered in nature, and it is expected to have a functional benefit as providing pH stress protection. This is stated as a mere fact (L309) but is important enough for explicit discussion and the trade-offs involved (e.g. more on the trade-off with oxygen), and perhaps also the reversibility of this process. Overall, comparing it to other pH stress response techniques are helpful. Many follow up experiments are possible to deepen the ecology/physiological side of the story, beyond the scope of this paper, but the authors could talk about this more and set the stage for future work.

Given the reviewer's recommendation, we now introduce the model in the text as a new section beginning on line 202, and present additional predictions in the new Fig. 3f. In addition, the Discussion

section was revised, and includes now expanded discussion of the pH/oxygen tradeoff (329-346) and of the dynamic nature of the condensed bacteria, as well as their expected reversibility (line 347-357).

3. “Chemotaxis is inherently single-cell” L25 (and L65). I know what the authors mean, but I disagree with this statement. Yes, the signal transduction happens in a single cell. But the network architecture can be shaped by selection acting on the collective motion. The lab of Thierry Emonet has produced some interesting papers on this. Concrete example: I think the very strong preference of E coli for serine and aspartate is not only determined by their metabolic values, but could be also be the result on selection of collective migration patterns driven by serine/aspartate uptake and self-generated gradient generation. It’s also not necessary for any of the claims made by the authors that bacterial chemotaxis is inherently single-cell.

Collective chemotaxis responses could indeed be a major driving force for the evolution of this system, yet, the basic ability of single cells to follow pre-existing gradients is still, in principle, independent of the surrounding bacteria. Anyway, to avoid these subtleties, we removed this statement from the abstract and from the introduction.

<u>And then some minor issues</u>:
L100. It was my understanding that motility buffer does not support growth for E coli RP437 because of an auxotrophic condition in that strain. It could be that it also does not support growth for MG1655, which is not an auxotroph, but in that case a reference would be needed.

The commonly used motility medium, in contrast to minimal medium, was generally optimized for cell motility rather than growth. Among other factors, it includes EDTA, which chelates essential trace metals, and lactate, which is not a very efficient carbon source. In any case, we tested the ability of cells to grow in motility medium, and, evidently, did not observe growth of MG1655 cells over the timescale of the experiments.

L78. The embedded population is referenced before the isolated population is discussed. So part of Fig 1A is not really used.
The aim of this cartoon is to clarify the principle distinction between ‘isolated’ and ‘embedded’ populations. Thus, while both terms are mentioned in the text, it seems to us that the order of their reference is not crucial.

Fig 4B add explicitly that increase in intensity is increase in pH
We now mention this in the text (lines 171-173) and in the figure caption (Fig. 3).

L243 Temperature comes in quite abruptly. Can the authors add a rationale or hypothesis of why they explored this parameter?
We revised this section (lines 269-282).

L282: weird formatting: “(Fig. 1-2), illustrated in Fig. 7A.”
Simplified (line 330).

Fig 1C it is my understanding that the complete area outside of the area indicated with a blue rectangle is hydrogel. Perhaps indicate this explicitly in the figure (e.g. make it darker for example).
Indeed. We have changed the colors so that the hydrogel looks slightly different.

I think it would be good to remind the reader explicitly about the differences in scale between AI-2 induced auto-aggregation and condensation. It may be required for a biology audience to more explicitly introduce and distinguish these two concepts as I anticipate for some readers this distinction is somewhat blurry.

The differences in scale between large scale cell 'condensation' and cell aggregates are best demonstrated in Fig. 6a, where both are directly shown. We now also stress the disparity in scale already in the last paragraph of the introduction (lines 81-85), as well as in lines 273-276.

We have also explicitly mentioned the AI-2-induced aggregates, which have a comparable scale to those shown here, in the introduction (lines 73-75) and now also in the discussion (lines 373-375).

I find the phrasing "The experiments done in Fig 1C" not very elegant. Just give the experiment a name and refer to it in the text.

Indeed, it is not very elegant, but we were mostly aiming for clarity here, trying to avoid confusion with other variations of the experiment. We now introduce these experiments as the 'thin-layer' experiments, but also refer to Fig. 1c in a few cases for clarity.

Addendum regarding the modelling part of the study:

Although the main contribution of this work is experimental, the numerical model adds quantitative insights, and hence also deserves more attention in the main text. The modelling aspect of the study seems overall carefully done but I have two sets of comments and suggestions:

-Section 1 seems in principle in line with previous descriptions of population-movement models. It would be very useful if the authors add the relevant references here (e.g. to Keller-Segel and important applications). However, the uptake term of protons is different from the typical uptake term that is used for many chemotaxis models. The shape of the term (linear) seems well constrained by data (Fig S7), but I had expected a Michaelis-Menten term here. The reason is that cells cannot simply absorb an arbitrary amount of ions (could the authors calculate how many ions each cell has to take up in this case?), and likely they need to use an enzymatic reaction to neutralize the protons after absorption. I expected this reaction at some point to saturate for a given amount of cells. Could the authors explain why this curve remains linear?

We now present the model in the main text and added relevant references there (line 202).

The form used here for the uptake is an approximation based on our experimental data (Fig. S7). Indeed, the uptake term is expected to saturate at higher proton concentration either due to a maximal velocity of proton neutralization (as in enzymatic reaction) or due to their finite capacity to passively absorb ions. However, few properties of this system should be noted.

(1) The external proton concentration is continually decreasing as the local environment of the cells becomes more neutral. Thus, the absolute rate of proton absorption is in fact decreasing. Therefore, if we consider Michaelis-Menten-like uptake term at the limit of $c \ll c_{sat}$:

$$r_H = \mathrm{r}_0 \frac{c^m}{c_{sat}^m + c^m} \approx \mathrm{r}_0 c_{sat}^{-m} \cdot c^m = \gamma c^m \quad (\gamma = r_0 c_{sat}^{-m})$$

Perhaps at lower pH (higher ion concentration) we could observe this saturation. We now discuss this in the SI.

(2) While cells cannot absorb arbitrary amounts of ions, the proton uptake is, evidently, linear in the cell density within the range tested here. This can be seen in Fig. S7, as data from different cell densities collapse onto the same curve once normalized by the cell density. This was plotted in the original figure but was not explicitly mentioned. We revised Fig. S7 accordingly. Thus, even cells at much lower cell density (OD 0.1) were able to neutralize the pH all the way to ~ 7.5 without saturating. Moreover, in the condensation experiments, the volume which needs to be neutralized is also shrinking over time as the population condenses, and thus, the time-integrated proton uptake is lower for condensing bacteria compared with dispersed cells.

(3) We did observe saturation of the proton uptake, but at low proton concentration: cells would not consume protons to a degree that makes the medium alkaline, and in fact, tend to saturate when the pH becomes higher than ~7.5. Yet, the bacterial pH taxis seems to weaken at pH values lower than that (6-6.2, see ref. 21). In fact, the dynamic range of the cells in our model as limited to pH 6 ($pK_{on} = 6$), and therefore we expect the saturation at high pH to be irrelevant for the solution. Finally, our numerical simulations also suggest that the pH within condensates does not approach neutrality.

-The entire method section in the supplement could benefit from editing, to bring it to the same level as the main text in terms of clarity. It should be at a level that another researcher (say another biophysicist) could reproduce the steps. Some examples: Section 1 has an unlabeled figure in the middle of the text. Section 2 and 3 are set up rather minimalistically and I think the authors ought to add more explanations and references here. The origin of the unnumbered equation in section 2 comes out of the blue (I believe it is just a phenomenological description). Also a sentence like “Eq. 2 was then used to estimate the chemotactic drift velocity” is too minimalistic. In Section 4, the parameter \alpha is not defined. Can the authors also provide an intuitive explanation what the parameter \epsilon means?

The supplementary part has been revised to enhance clarity. In particular, figure labels and captions were added, equations were numbered and introduced more carefully, and additional explanations were provided.

REVIEWERS' COMMENTS:

Reviewer #1 (Remarks to the Author):

None

Reviewer #2 (Remarks to the Author):

The authors have addressed all my concerns and I appreciate the effort of the authors to perform additional experiments and clarify their manuscript. They present it in a modest way, but their experiment at pH 9.6 is very impressive: It shows that the condensation effect is not specific to low pH but a general feature of the E coli response to uniform environments with a deviating pH! The additional explanations regarding the model in the main text serve this paper well. I highly recommend it for publication.