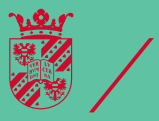

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Regulation of Indole Production Kinetics in *E. coli*

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1. Introduction

1.1 Indole as trigger of dormancy

Cells actively uptake metabolites from the external medium in order to build biomass during the process of growth. This growing phenotype is predominant when conditions in the surrounding environment are favorable for cell division and a non-growing prevail during nutrient starvation. However, bacteria can display a dormant or persister phenotype even when the conditions are favorable to growth (Figure 1)[19,33]. The bioclinical relevance of this state is that it has been reported that most of the surviving cells to an antibiotic treatment are in a non-growing persister state[41].

To switch between growing and dormant states, different mechanisms have been described which can be divided into responsive switching, occurring as a direct response to an outside signal detected by a sensing mechanism, and spontaneous stochastic switching[22]. Stochastically switching means that even in environments where the conditions are adequate for growth, a certain number of cells do not follow the majority behavior but enter a dormant state (Figure 1)[19]. A good example of the underpinning mechanisms are toxin-antitoxin modules[10,19,26,31].These are comprised of two components: a gene encoding for a "toxin" that leads to persistency, and an "antitoxin" that blocks toxin activity allowing cells to growFor example, hokB is a stable toxin that causes persistency via membrane depolarization when active, whilst *sokB* codes for an antisense RNA that blocks *hokB* transcription leading to its degradation. Under normal growth conditions, sokB repression is predominant in most cells allowing growth but a small subpopulation present a overexpression of *hokB* that drives cells to a dormant state[41]. On the other hand, when cells receive information from environment, they trigger signalling cascade that leads to a phenotypic change. A well-known example of responsive switching is the transition between exponential phase to stationary phase. As a signal-driven strategy, cells are able to sense a decrease in nutrient influx to take further action controlling the entry into dormancy at the beginning of stationary phase[14].

It has previously been described that during the entrance of *E. coli* to the stationary phase, cell-associated indole (see section 3.3) rapidly increases to control this transition. This phenomenon is called the "indole pulse"[11]. Although some researchers assert that indole acts through a protein receptor[34], the Summers group has developed the theory that indole acts by disruption of membrane potential (see section 3.3). In this scenario, indole acts primarily as an ionophore, allowing protons to cross the cytoplasmic membrane[4]. This consequently collapses $\Delta\Psi$ and blocks cell division[4], leading to a low metabolic state. Indole production not only correlates with entry into persistency but also helps bacteria survive against adverse conditions, such as exposure to antibiotics[32, 24, 32] by up-regulating expression of various drug exporter genes[15]. In addition, stopping growth while there still exists a reserve of unused nutrients increases cell survival in prolonged stationary phase. Furthermore, this signal-driven response allows emergence of population behaviors like the generation of biofilm protective structures.

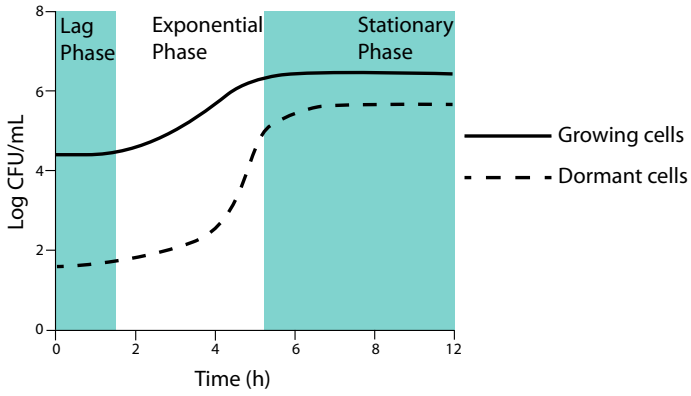


Figure 1: Growth-stage dependence of persistent cell formation in *Escherichia coli*. Cells divide when conditions are favorable (exponential phase), so the number of dormant cells is low and mainly stochastic. However, at the stationary phase the dormant cell fraction is higher. We hypothesize this change in frequency could be triggered by metabolic signals as detailed in section 3.3. Figure adapted from ref. 19. CFU, colony forming unit, an estimator for bacterial population size.

1.2 Indole and membrane potential regulation

Indole is a heterocyclic aromatic molecule with multiple signalling roles. It is produced from tryptophan by the enzyme tryptophanase (*tnaA* in *E. coli*)[2]. Tryptophanase has highly complex regulation based on two different promoters, the presence of high concentrations of tryptophan and the polymerization of the enzyme[24] and it is present in hundreds of species, which means that modulating $\Delta\Psi$ via indole production could be a conserved stress response strategy. Work on *tnaA* mutants has revealed that indole promotes biofilm formation[8], virulence and responses to stresses, including antibiotics[40]. In recent years the biomedical significance of indole has increased due to its role in mediating inter-kingdom signalling between enteric bacteria and their mammalian host, influencing cellular health and physiology[4].

The molecular mechanisms underpinning indole signalling are poorly understood. Although there is currently no direct evidence, it is often assumed that indole exerts its effect by binding to a protein receptor that, directly or indirectly, modulates transcription of relevant sets of genes[34]. However, The Summers group has developed a unified theory of indole signalling that proposes all of the outcomes of indole signalling arise via modulation of the electrical conductivity of the cytoplasmic membrane. They have shown already that the inhibition of cell division can result from an indole-induced reduction in electrical potential across the cytoplasmic membrane[4]. In collaboration with Ulrich Keyser (Deptartment of Physics, University of Cambridge) it was elegantly shown that indole acts as an ionophore, allowing protons to cross freely the cytoplasmic membrane[2,4]. The consequent decrease in the electrical potential across the membrane inactivates the Min site selection system, preventing the localization of FtsZ and the formation of the "divisome" complex[4] and blocking cell division[2,4].

However, up until now, the way in which the metabolism communicates with the membrane potential remains unknown. Most of the approaches to answer this question depart from the classic view that "a gene controls a phenotype", hence most of the analysis seek differences between a tryptophanase mutant and a wild type. Nevertheless, tryptophanase has a really complex regulation based on two different promoters, the presence of high concentrations of tryptophan and the polymerization of the enzyme (Figure 2)[9]. Here, we have to bear in mind he pervasive effect of membrane potential that affects some of the most important

process in cell metabolism such as metabolite transport across the membrane or ATP formation.

Therefore, here we propose to characterize the dynamics followed by *E. coli* in order to regulate the production of indole. It is known that in this organism there are two promoters controlling the activity of *tnaA*: Crp and TorR-P (Figure 2). In addition, it has been characterized that indole is mostly a secondary metabolite produced during the entrance into the stationary phase in a pulse-like kinetics. Here, we propose to explore in a deep level of this dynamics. We use an multi-perspective approach based on direct indole measurement in supernatant, flow cytometry data analysis using the tagged strain *tnaA*::*GFP* and microscopy observations at different cell grwoth stages of the same strain to describe the response of *E. coli* under different conditions. Here we found that the responsive space of *E. coli* is wider than it was previously reported, which means *E. coli* can display different responses in different circumstances. Moreover, we will also propose a plausible model and the derived questions from it.

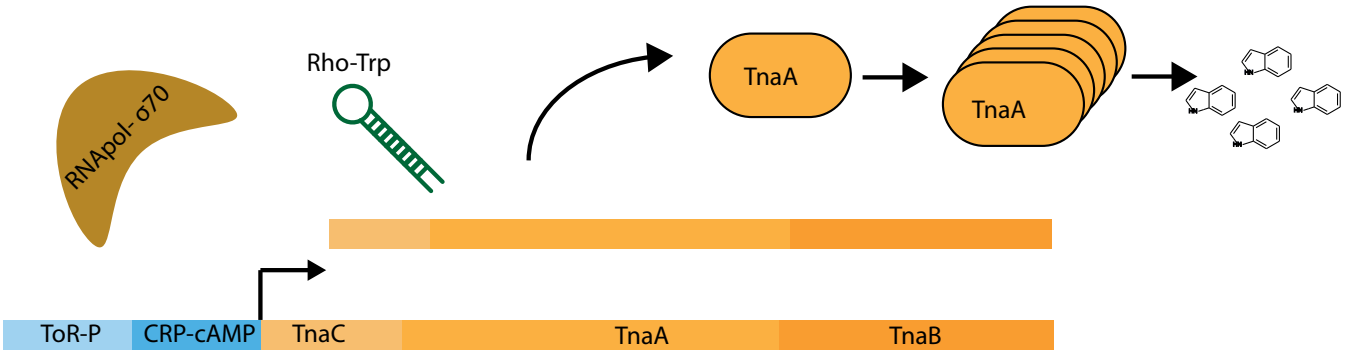


Figure 2: The *TnaCAB* operon codes for two key enzymes in tryptophan metabolism, including trytophanase. There are two promoters that positively regulate the expression of the operon (blue). Crp stands for Catabolite Repression Protein, which regulates the expression of over 180 gene, many of these genes are involved in the catabolism of secondary carbon sources. TorR is a transcriptional DNA-binding dual regulator being a positive regulator for genes related to Nitrate respiration and alkaline stress. Also, attenuation of *TnaCAB* occurs via ribosomal pausing at the tail end of the *TnaC* leader sequence. Tryptophan prevents premature termination via Rho-Blocking antitermination. The tryptophanase is transcribed as monomer but it is actually active when it conforms tetramers/aggregates. All the references were taken from [9]

2. Impact of this work

2.1 The persistence phenotype is a public health problem

The power of antibiotics to cure illness is taken for granted by most people, but as World Health Organization President Margaret Chan stated, their effectiveness is rapidly decreasing. Infectious diseases are now the second leading cause of death worldwide[35] as extensive antibiotics use has had a profound impact on bacterial ecology and presents a striking example of evolution. Approximately 0.1% of an isogenic bacterial population display tolerance to prolonged treatment with high doses of bactericidal antibiotics[19,26,31]. TIn biomedicine, these cells are termed persisters and are behind chronic infections such as tuberculosis (TB), candidiasis, and cystitis[12]. In addition they serve the as platform for antibiotic resistance[6, 25]. The archetypal example of a persistent microorganism is *Mycoplasma tuberculosis*; the cause of TB. Although TB is predominantly a disease of poverty, with over 80% of cases occurring in Africa and Asia, globally in 2014 there were an estimated 9.6 million new cases and 1.2 million deaths[44]. It is estimated that 3.3% of new diagnoses, and 20% of previously treated cases are due to multi-drug resistant *M. tuberculosis*[43,47] .

In the Summers' group (University of Cambridge), we believe that genetics constitutes the key tool to shed light on persistency regulation. From the genetic point of view, persisters are not mutants. Rather, they are epigenetic variants distinct from actively dividing cell. These cells have entered a metabolically quiescent state where they neither grow nor die when exposed to lethal concentrations of antibiotics[10,13,19,26,31]. . As such, the mechanism that leads to persistency is one of the most pressing questions in clinical microbiology.

However, dormancy is a phenotype that could be reached for several different mechanisms and we consider that in a short future it will be more relevant the understanding of the signal-driven mechanisms. More precisely, we have focus our efforts on indole in view of the fact that Today, the molecular mechanism of indole action remains poorly understood although the literature offers a number of unsubstantiated models. Here, the value we want to give with this project is to describe the clear mechanism between the cell metabolism and the indole production kinetics.

3. Results

3.1 Indole production is related to carbon source

We had previous evidence that indole production might occur during entry into stationary phase following a pulse-like kinetic in LB[11]. Moreover, it was also reported that an increase of initial glucose concentration lead to a significant delay on the indole pusle[12]. Consequently, our initial question addressed the hypothetical connection of carbon metabolism with the regulation of indole production. The available information in the databases revealed that this connection could happen via Crp[17] (Figure 2). Therefore, we deduced that the indole response at the entry into the stationary phase might be the result of Crp up-regulation caused by a decrease in glycolytic flux[14, 20].

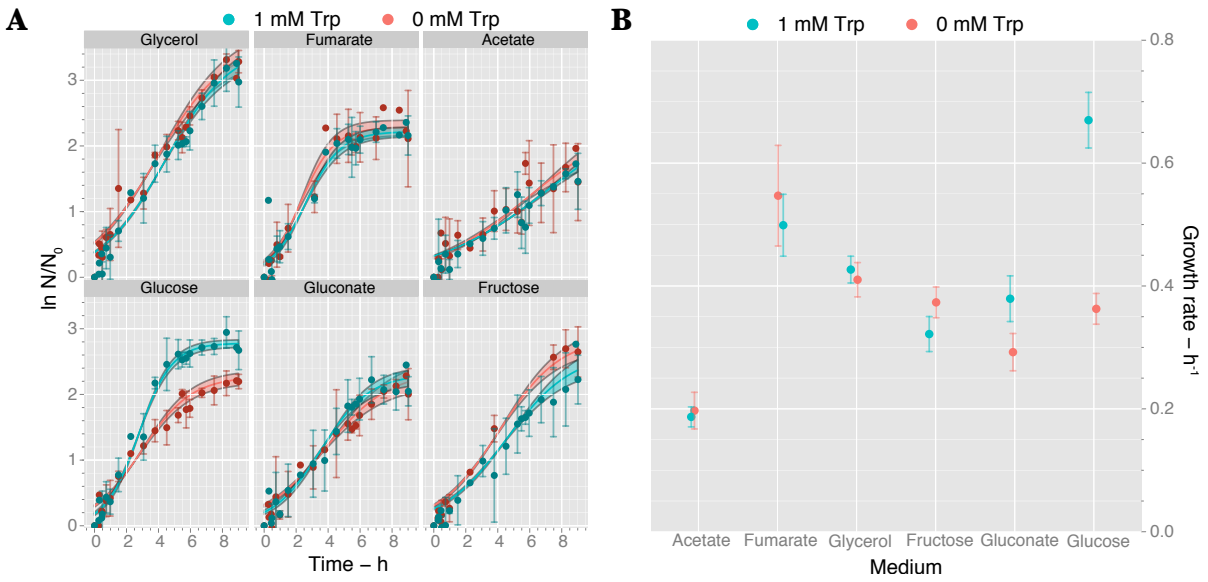


Figure 3: Fundamental behavior of *E. coli* in minimal medium plus carbon source and 1 mM Trp (see Materials & Methods). **A -** *E. coli* growth during 9 h culture 37°C. The change in biomass is estimated using Optic Density at 600 nm (OD). The OD measurements are expressed as natural logarithm relative to the initial OD (N_0) as described by Zwietering[48]. The dots represents the empirical observations and the line represent the fitted values. Error bars represent the standard error of 5 replicates and the colored area the values inside the standard deviation of the calculated parameters. Regression was made with GroFit[18]. **B -** Estimated maximum growth rates (μ_{max}) in medium with different carbon sources. Error bars indicate the standard deviation of the estimated parameter.

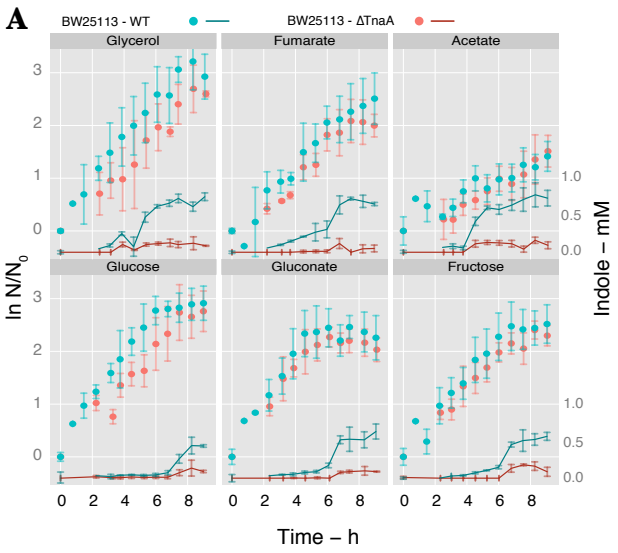
To approach this question we used different carbon sources to stimulate different glycolytic fluxes in *E. coli* and accordingly, different Crp pattern responses. We selected a total of six carbon sources based on two criteria: the maximum growth rate with that carbon source, and the "fermentability" of the carbon source. Thus, we classified our carbon sources into two categories: Gluconeogenic (Glycerol, Fumarate and Acetate) for those that, independent of their growth rate, cannot be passed through glycolysis and Glycolytic (Glucose, Gluconate and Fumarate) for those that are preferably fermented[14, 45]. The underlying idea is that high growth rates should be inversely correlated with Crp up regulation, which in turn will trigger indole production.

Our data showed that the highest maximum growth rate (μ_{max}) is in glucose (Figure 3A & Figure 3B). This agrees with the previous results because presence of glucose in the medium leads to a delay of indole production[12]. We also expect a similar behavior in gluconate and fructose. On the other hand, the lowest μ_{max} value was obtained when acetate was used as a carbon source (Figure 3A & Figure 3B). Now, our prediction says that acetate will trigger a stronger indole response, but due to the fact that fumarate and glycerol are also gluconeogenic, they will also display strong indole responses.

Furthermore, we performed indole measurements from cultures under these conditions, so we established cultures of *E. coli* WT and Δ*tnaA* (See Table 1) and tracked them by taking samples each 45 min during 9 h. All the gluconeogenic carbon sources produce a significant indole concentration (>0.1 mM) at an earlier stage (<6 h) and produce indole until tryptophan depletion, reaching 1 mM maximal (Figure 4A). Moreover, it could be that due to low initial cell number, cells are producing indole but the total amount remains below the detection limit. In terms of glycolytic carbon sources, in all cases a delay of the indole response (>6 h) was observed until the culture approached the stationary phase. Before cells started to produce indole in glycolytic carbon sources, the cell number was high enough so here it seems reasonable to discard the hypothesis of cells producing indole below of the detection limit. Figure 4B shows that the relationship between OD and indole increases quasi-linearly for the gluconeogenic carbon sources and exponentially in the case of glycolytic carbon sources. This second response has been previously described as indole pulse response[11].

Finally, we performed a clustering algorithm in order to assess whether the classification gluconeogenic/glycolytic was artificial or instead it has a biological implication on the cells behavior. For that purpose, we took the average indole production throughout the time culture to perform a cluster algorithm based on Euclidian Distances (Figure 34C). The analysis revealed that *E. coli* supplemented with glucose, fructose or gluconate displayed a similar response as compared with acetate, fumarate or glycerol. Nevertheless, based on our hypothesis, we expected glucose and acetate to be located on opposite extremes of the cluster tree.

Our next question was to assess whether this difference in indole production was mediated through changes in *tnaA* transcriptional regulation. For that we used an approach based on fluorescence and flow cytometry.



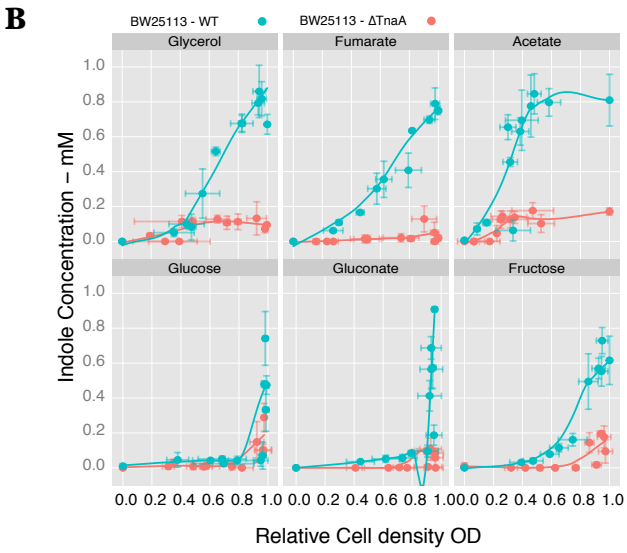
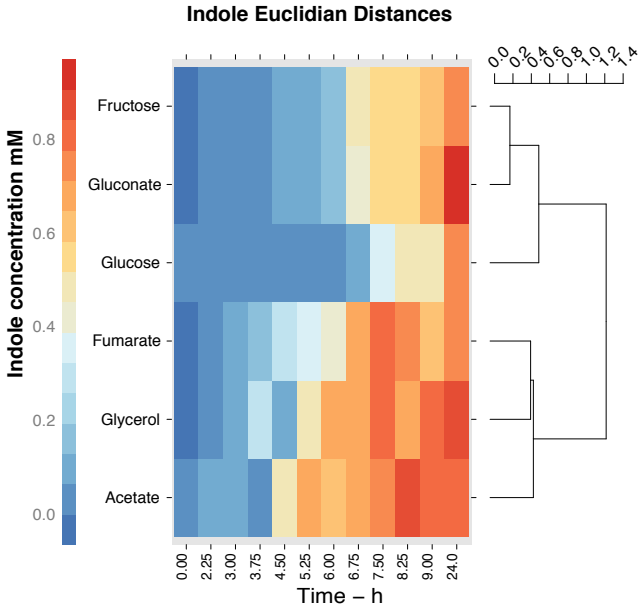


Figure 4: Relation between cell growth, carbon source and indole production. Glycolytic carbon sources lead to a delay in indole pulse and gluconeogenic carbon sources trigger an earlier indole response. **A -** The first panel shows the mean increase of biomass (dots) relative to initial OD (0.05). The lines shows average change of indole concentration for each time point expressed in mM. Red color was used to designate *tnaA* defective mutant and green for WT. Error Bar indicates the standard deviation. **B -** Relation between indole concentration with relative biomass to the maximum. Red color was used to designate *tnaA* mutant and green for WT. Error Bar indicates the standard deviation. **C -** Color plot describing the average change of indole concentration of WT cultures with different carbon sources and clustered by complete clustering linkage. On the left, the cluster tree with the relative distance units scales on top.

C



3.2 Indole Production kinetics is coupled with *tnaA* expression kinetics

The direct measurements of indole in supernatant suggested a difference in production kinetics depending on the carbon source. In case of gluconeogenic carbon sources indole was produced early on stage of the culture. In addition, previous reports have also shown that different carbon sources lead to changes in Cra activity[14,20,21], thus changes in Crp activation levels[14]. Due to the presence of a Crp-cAMP binding box in the promoter region of *tnaA*, we asked whether the different indole production kinetics was caused by changes in the expression pattern of *tnaA*. In order to resolve this question, we employed the same experimental conditions used for indole measurements (see Materials & Methods), but using *E. coli* BW25113 *tnaA*::*GFP*. This allow use fluorescence as a proxy of *tnaA* expression levels. Also, we use as a control media lacking tryptophan, on the ground that it has been reported that in the absence of tryptophan indole is not produced[29].

Fluorescence data from flow cytometry revealed that cells growing on gluconeogenic carbon sources express *tnaA* early on during growth(<2 h) following a sigmoidal dynamics and reaching high expression levels (≥4 Log Arbitrary Units or A. U.) (Figure 5). Additionally, in the presence of acetate, expression levels where the highest as predicted by our theory. The data from glycolytic carbon sources was more difficult to analyze. There, we found that the expression around 3.5 Log A.U. overlaps with the indole pulse as well as with the stationary phase. We also observed in glucose an intermediate fluorescent stable state (Figure 5, around 2.6 Log A.U.). In the case of gluconate the intermediate state was produced between 1 h to 5 h with a higher fluorescent intensity close to 3.0 Log A.U. Finally, in fructose that state was barely appreciable.

Consequently we asked whether *tnaA* is present in the cell in two stable forms: one active and another conformation that logically will be inactive.

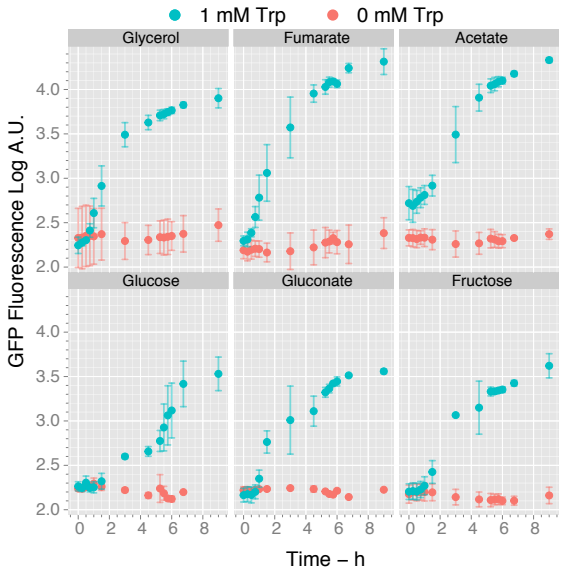


Figure 5: Flow Cytometry data. Dots shows the average value of the fluorescence intensity logarithm. Green color indicates cultures supplemented with 1 mM Trp and red color indicates culture with 0 mM Trp. Error bar indicates standard deviation from the mean of three biological replicates.

3.3 Changes in *tnaA* expression Are related with changes in *tnaA* structure

The evaluation of the flow cytometry data revealed that for glucose, gluconate and more moderately fructose the fluorescent signal comes in two stages (Figure 5). One early low fluorescent stable level at a time when there is no significant indole production and a second stage that overlaps in time with indole production. Regarding this, previous reports have demonstrated that tryptophanase activity is related to a foci-like structure[28]. We therefore, investigated the possibility that the intermediate stage could be caused by an inactive conformation of tryptophanase. To elucidate this question, we prepared microscope slides from the same samples used for the flow cytometry (see Materials & Methods).

The data revealed that all cells under our conditions are able to display three different phenotypes regarding TnaA::GFP: no fluorescence (Figure 6A), dispersed fluorescence (Figure 6B) and localized foci (Figure 6C). We decided to perform an study these three populations during culturing time. We observed that cells started with no fluorescence, but rapidly the difference emerges between the gluconeogenic and glycolytic condition (Figure 6D). When cells are exposed to glycerol, fumarate or acetate, they tend to form focial structures very quickly (<3.75 h). In the case of glucose and fructose major population of cells with fluorescence emerged during the first 6 h but no foci was reported (Figure 6D). It is important to note that gluconate and fructose present an higher degree of heterogeneity as compared to other cases. What is clear for all conditions is that during the late growth stages (>6h) the dominant phenotype is cells presenting foci structures. Another remarkable fact is that the foci intensity is brighter when the carbon source is gluconeogenic (data not shown). This result dovetail with our previous insights.

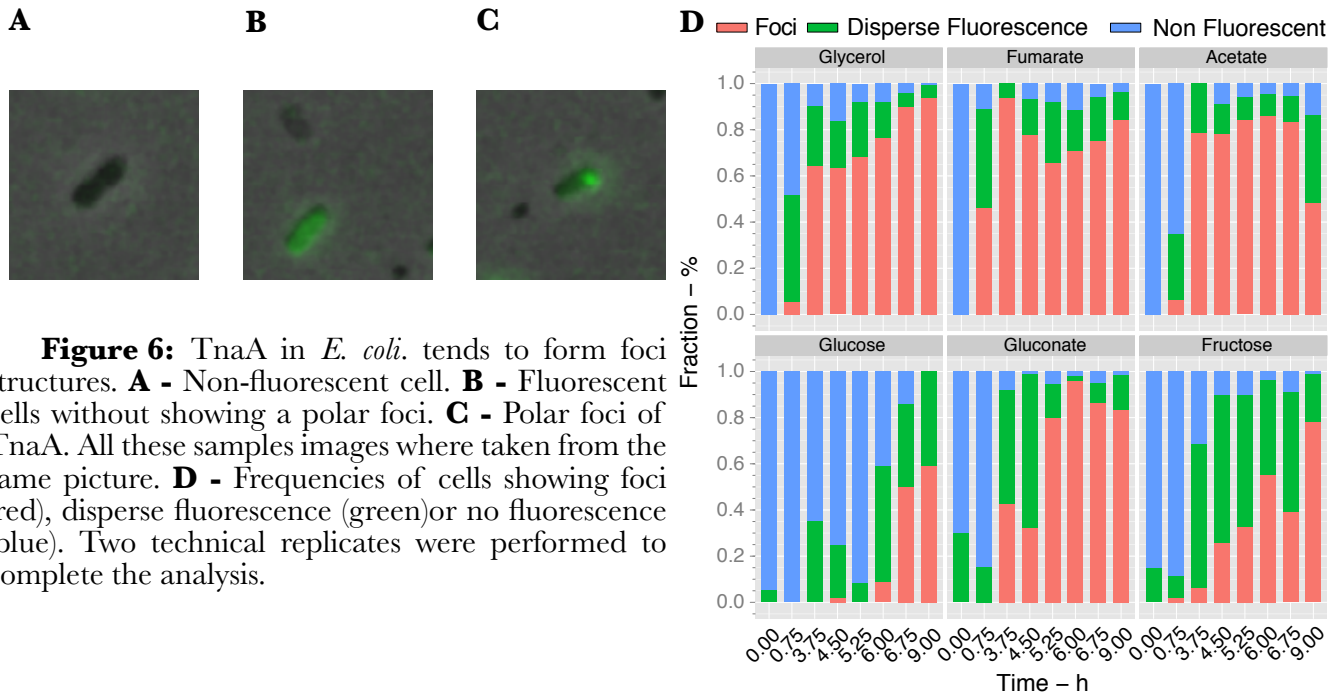


Figure 6: TnaA in *E. coli*. tends to form foci structures. **A -** Non-fluorescent cell. **B -** Fluorescent cells without showing a polar foci. **C -** Polar foci of TnaA. All these samples images where taken from the same picture. **D -** Frequencies of cells showing foci (red), disperse fluorescence (green)or no fluorescence (blue). Two technical replicates were performed to complete the analysis.

All together our data suggest that when *E. coli* is exposed to oxidated carbon sources, the preferred behavior will be to produce indole as long as tryptophan is available. Moreover, it has been shown that in the presence excess of glucose, *E. coli* is more prone to use acetic fermentation and only switch to full respiration when it approaches the stationary phase [14,45]. Therefore, we postulate here that a switch between fermentation will pose a high impact on the proton balance of *E. coli* due to the sudden activation of the respiratory chain. Because indole has been reported to act as a ionophore, we decided to explore the possibility that indole might modulate the membrane potential in *E. coli*.

3.4 Ttryptophanase expression might be related with changes in membrane potential

It has been reported that the external addition of indole permeabilizes the *E. coli* cytoplasmic membrane to protons either in live cells or in synthetic lipidic spheres[5]. However, little is known about the innate mechanism of action of indole in live cells. Therefore, we designed a experiment in order to describe the relation between indole production and membrane potential *in vivo*. We used again *tnaA*::GFP strain (see Materials & Methods) taking fluorescence intensity as a proxy of indole production and the dye Oxonol-VI as membrane potential probe[4,30,39] we used the same experimental set up as before (see Materials & Methods) to grow cells in different carbon sources and supplemented with or without 1 mM Trp. Oxonol-VI has been reported as a fluorescent probe to membrane potential[30].

These results obtained here are highly preliminary and it is difficult to draw firm conclusions. A first analysis showed that cell membranes depolarizes steadily in all carbon sources as cells approach the stationary phase (Figure 7A). This increase is positively correlated to an increase in *tnaA*::GFP fluorescence (Figure 7B). In addition, we observed that the cells could do this regardless of the presence of tryptophan, thus without being influenced by indole production. Furthermore, all carbon sources tend to reach some stable point from which fluorescence does further increase (Figure 7A).

However, it has been discussed that Oxonol-VI fluorescence could be correlated not only to membrane potential, but also to cell size, as bigger cell could take up more dye molecules and, hence give brighter signal. Therefore, we applied a mathematical correction using the intensity from the forward scatter channel (generally abbreviated as FSC), whose intensity is correlated with cell size[42]. Thus we compensate the potential noise from the cell size (see Materials & Methods)[39]. This derived parameter showed that Oxonol-VI fluorescence does not correlation in any condition with time. It is highly probable that this effect is an artifact, it has repeteadely reported that the membrane potential changes in the transition exponential phase/stationary phase[36]. Moreover, this artifact could be caused by the use of formaldehyde to block the reaction and fix the cells because formaldehyde creates pores in the membranes that cause membrane depolarization[7]. For this reasons, we consider it necessary to have a replication with an independent method in order to confirm the relation between indole production and membrane potential.

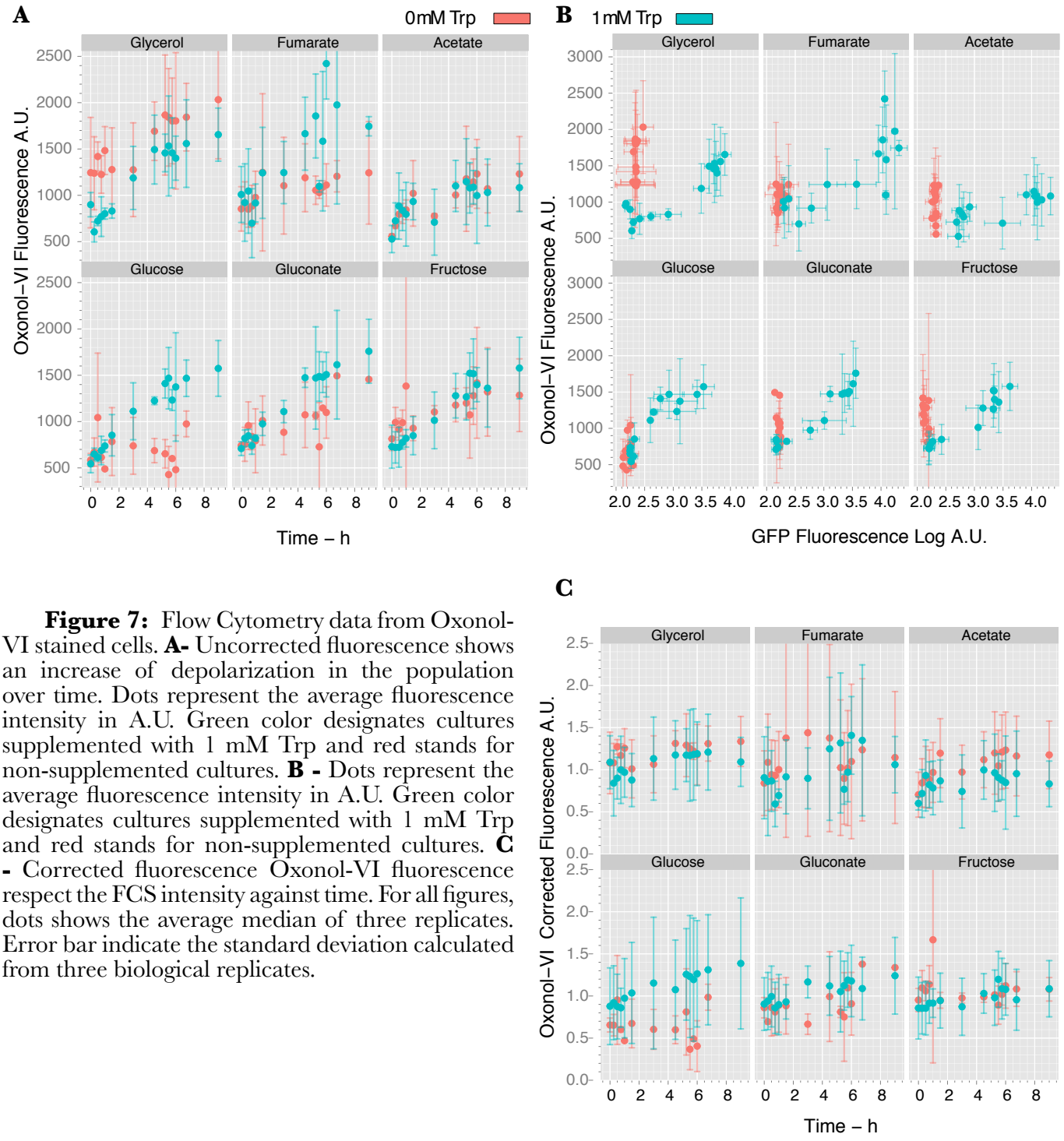


Figure 7: Flow Cytometry data from Oxonol-VI stained cells. **A-** Uncorrected fluorescence shows an increase of depolarization in the population over time. Dots represent the average fluorescence intensity in A.U. Green color designates cultures supplemented with 1 mM Trp and red stands for non-supplemented cultures. **B -** Dots represent the average fluorescence intensity in A.U. Green color designates cultures supplemented with 1 mM Trp and red stands for non-supplemented cultures. **C -** Corrected fluorescence Oxonol-VI fluorescence respect the FCS intensity against time. For all figures, dots shows the average median of three replicates. Error bar indicate the standard deviation calculated from three biological replicates.

4. Discussion

4.1 Mechanism of Indole regulation

In the next decades, public health institutions will have to deal with new challenges such as persister infections. Unfortunately, the Golden Age of Antibiotics is already part of history[35]. Therefore, it is going to be necessary to employ new tools to success in the health battle and for that purpose it will be required a complete understanding on dormancy. With this work we have taken one small step in order to describe the mechanism used by microorganism to enter into a dormant state. While most of the literature is focused on the stochastic dormancy, here we have extended the characterization of one novel mechanism. Recent studies have demonstrated that indole is produced at the entry to stationary phase and that the presence of glucose is a very strong repressor of indole production[11,12]. The work presented here revises that model to include other carbon sources revealing that the kinetics of indole production by *E. coli* is affected strongly by the prevailing carbon source. More precisely, we described two radically different responses depending on the type of carbon source, glycolytic or gluconeogenic (Figure 4). Interestingly, when *E. coli* is exposed to glycolytic carbon sources, the indole pulse is delayed until the culture approaches the stationary phase, whereas in the case of gluconeogenic carbon sources indole production starts very early growth stage. Here, WT could have a better control over its membrane potential than a Δ*tnaA* strain and consequently WT could posses a better control of transport across the membrane, including the xenobiotic exporter pumps.

Moreover, we have shown that this change in indole production is probably caused by a change in the pattern of expression of tryptophanase. Again, in the case of glycolytic carbon sources such as glucose, there is a delay in the high level production of tryptophanase. What is more, during this delay, cultures show a "mild" tryptophanase expression where the enzyme appears not to be active since little indole is produced. On the contrary, in conditions where *E. coli* is supplied with gluconeogenic carbon sources the expression of tryptophanase rises rapidly and from the beginning reaching a saturation point of expression (Figure 5).

Furthermore, we speculate that this change in tryptophanase level and activity is linked with a change in distribution since it has been reported that the active form of tryptophanase occurs when the enzyme is condensed in a foci structure. The microscopy analysis of cells in all our conditions, confirmed that the increase in tryptophanase also entails a change of enzyme location from cytoplasmic diffuse to condensed foci. Accordingly, we reported predominant frequencies of cells with foci in all stages when gluconeogenic carbon sources are used but only at late stages when glycolytic carbon sources are used (Figure 6).

In addition, we give certain evidence that there might be an association between indole production in a growing culture and a change in the membrane potential of cells, which is in agreement of previous work[4,36]. We first reported a positive correlation (Figure 7), but then decided to do a deeper analysis that contrasted with our original results. This second analysis points in the direction of a relation between our proxy to membrane potential and tryptophanase expression and moreover it suggest that membrane potential is

highly stable in cells during culture time (Figure 7). However, we take this last result carefully on the light that other reports indicate that the membrane potential is different between stationary phase and exponential phase. Thus, we believe it could be an artifact caused by the use of formaldehyde[7] (see Materials & Methods) as it has been demonstrated that formaldehyde interferes with membrane permeability, thus membrane potential of cells. We think that a further work it is necessary to evaluate of this membrane potential of cells growing on medium with different carbon sources.

4.2 Proposed model of Indole Production kinetics

From the departure point that indole synthesis by *E. coli* is triggered by glucose depletion and that glucose blocks indole production we extended the model to other carbon sources. This allow us to shift the model from a metabolite-specific response to a parameter-broad response. We theorize that *E. coli* could be using key parameters from the glycolytic flux or TCA-flux[14,20,21] in order to fine tune its behavior. From a systemic point of view this might be more efficient, allowing *E. coli* to adjust the phenotypical response to a wide variety of conditions, including those that are not typical to its natural environment. Thus, we propose a plausible model to explain the kinetics of indole production based on the available metabolomic and genomic data which is coherent with our empirical results (Figure 8). We propose that *E. coli* uses the two key steps of glycolysis as "probe" of the glycolytic flux. Thus, when the glycolytic flux drops, *E. coli* tends to deviate the flux to the TCA and thus increasing the respiration rate. Along with this change, the system also triggers the production of TnaA.

Despite our initial results, we still defend the idea that the key role of indole is to modulate the membrane potential in *E. coli*, as it has already been shown that addition of indole depolarizes cells. Thus, it might be that when cells switch from fermentation to respiration at entrance of stationary phase, they use indole to regulate the change in membrane potential[36]. This could be the preferred way or it could be that microorganism have more than one way to control their proton balance across the membrane. It is also interesting to ask, why natural selection has evolved a mechanism to permeate membranes to protons since that *E. coli* oxidice carbon sources to pump out of membrane protons that theoretically could be converted into ATP by the ATP synthase.

4.3 Further work

The model described in Figure 8 could be the starting point for some simple experiments with a high potential impact. Here we have used different carbon sources to trigger or modify the indole production kinetics. Then, it would be interesting to repeat the same experimental setup but using different carbon concentrations. Our prediction is that an increase of the glycolytic carbon sources will significantly delay the indole production when the increase of gluconeogenic carbon sources will barely produce any change.

Secondly, our model for indole production assumes that the carbon metabolism communicates with the genome via *crp*, and that this is the final responsible of *tnaA* expression. It will be interesting to correlate the expression of *crp* with *tnaA*. This could be done in two ways. On one hand, using GFP expression as a proxy of gene expression is a well established method to estimate relative gene expression. On the other hand, the use of quantitative PCR represent a much more precise tool to describe gene expression. These two simple experiments could cover big part of the exposed model.

In a later stage, it will be interesting to tackle the role of TorR-P in the expression of *tnaA*. Indeed, it has been already shown that *tnaA* can be expressed independently of the cAMP[27] path. For this, the key experiment should play with the condition of different carbon sources plus different pH. It has also been reported that TorR-P has a role in anaerobic fermentation, so this condition needs also to be explored, initially by having cultures in anaerobic condition and measuring indole.

Finally, we have no clue of what might be the intra-cellutar downstream effect of indole. Previously, it has already attempt to test the effect of external addition of indole, showing few genes to be affected. But, then, how a big phenotype change as dormancy is achieved by affecting only few genes? On top of that, it is also compelling ask that since tryptophanase is not an essential gene, how the cell can override its deletion? How they can regulate with membrane potential transitions when this tool is not available?

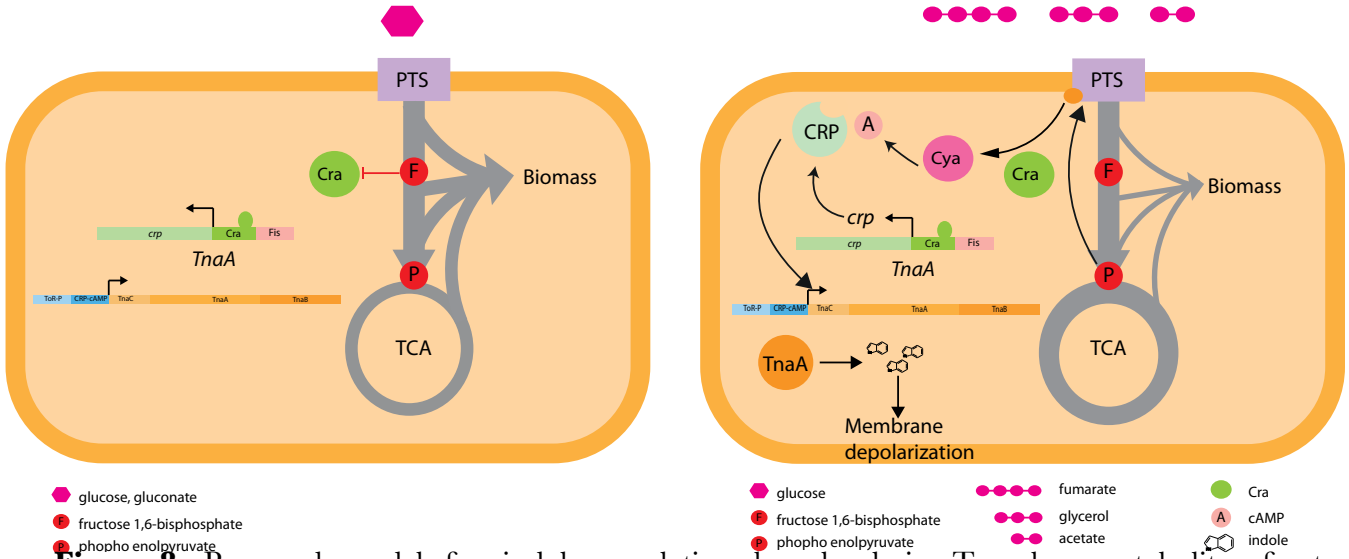


Figure 8: Proposed model for indole regulation by glycolysis. Two key metabolites fructose 1,6-bisphosphate (FBP) and phosphoenol pyruvate (PEP). When there is high glycolytic flux (i.e. in presence of high concentrations of glucose), there is a high concentration of FBP blocks transcription factor Cra and high PEP levels prevent the phosphorilation of the EIIA system, thus blocking Adenilate clyclase (Cya) activity. However, when the glycolytic flux is low (i.e. low glucose concentration or gluconeogenic carbon sources), the levels of FBP decreases triggering Cra activity, thereby Crp expression [20]. In addition, a drop of PEP, lead to EIIA phosphorilation, which in turns activates Cya[2]. These combined lead to the formation of Crp-cAMP complex that enhances expression of Tna in presence of tryptophan.

5. Materials & Methods

5.1 Indole measurements

All experiments were performed using M9 minimal medium, which was prepared as previously described[21] or Luria Bertany broth (LB) that was autoclaved and then filtered. The medium was supplemented with a carbon source to a final concentration of 1 g/L in the case of glycerol, fumarate and acetate or 0.5 g/L for glucose, gluconate and fructose. The carbon source stock solutions were made by dissolving the carbon source in demineralized water, adjusting the pH to 7 with NaOH or HCl, and filter sterilizing.

Cells where first inoculated from a LB-agar (1.5%) plate into 10 mL liquid LB and placed at 37 ºC 300 rpm (Multitron Standard Incubator, Infors). Next day the culture was diluted into a 50 mL Erlenmeyer containing 19 mL of M9 medium plus 1 mL of the carbon source stock to the indicated final concentration and incubated at 37 °C, 300 rpm for 8 h. Finally, the culture was diluted 1:50 in the same medium. Finally, next day the culture was diluted into the same medium at an OD of 0.05. In the indicated case tryptophan was added up to a concentration of 1 mM. The strains used in this work are detailed in Table 1.

Table 1: List of E. coli strains used in this work.

Strain	Phenotype	Reference
BW25113	$lacI^q$, $rrnB_{T14}$, ΔlacZWJ16 hsdR514, $\Delta araBAD_{AH33}$, $\Delta rhaBAD_{LD78}$	Reference [1] & reference [46]
BW25114 *tnaA*::GFP	$lacI^q$, $rrnB_{T14}$, ΔlacZWJ16 hsdR514, $\Delta araBAD_{AH33}$, $\Delta rhaBAD_{LD78}$, *tnaA*::GFP	Reference [11]
BW25113 Δ*tnaA*	$lacI^q$, $rrnB_{T14}$, ΔlacZWJ16 hsdR514, $\Delta araBAD_{AH33}$, $\Delta rhaBAD_{LD78}$, Δ*tnaA*::Km^R	Reference [1] & reference [46]

5.2 Strains and culture growth conditions

To assay indole in culture supernatants, a sample (1 ml) from a growing culture was removed, the OD_{600} measured and cells harvested by centrifugation at 11337 x g for 15 seconds (Eppendorf Minispin microfuge). The supernatant was removed and assayed: 300 ml of Kovacs Reagent (10 g of p-dimethylamino-benzaldehyde dissolved in a mixture of 50 ml of HCl and 150 ml of amyl alcohol) was added to the supernatant and incubated for 2 min. A 50 ml portion was removed and added to 1 ml of HCl- amyl alcohol solution (75 ml of HCl and 225 ml of amyl alcohol). The absorbance at 540 nm was measured (Gene Quant 1300,

GE Spectrophotometer). The concentration of indole in the supernatant was calculated using a calibration curve.

5.3 Flow Cytometry & Oxonol-VI staining

Cells cultures were grown as described before using the *tnaA*::GFP strain. To estimate transmembrane potential, a sample (1 ml or 0.5 mL) from a growing culture was removed, the OD_{600} measured and samples were frozen at -195 ºC by immersion in liquid nitrogen and then stored at -25 ºC until further analysis. When all samples were collected, the analysis was performed. Samples were thawn at 37 ºC. Then Oxonol-VI was added to a final concentration of 10 µM from a 1 mM stock ethanol. Samples were incubated for 10 min before the addition of formaldehyde to a final concentration of 1%. Flow cytometry was performed on a Cytek DxP8 FACScan (Cytek).

Fluorescence was excited by a 100 mW laser at 561 nm and 30 mW at 488 nm. Fluorescence was measured through a 615 nm emission filter of 25 nm width and 530 nm filter with 30 nm with. For each sample, 100,000 events where recorded at a rate between 5,000-10,000 events per second using FCS as trigger. We use a Photon Multiplier Gain of 350 for Side Scatter (SSC), 500 for 530 nm signal and 350 for 615 nm signal. Data was analyzed with the R package "FlowCore" and "FlowViz"[37].

5.4 Microscopy and & Image Analysis

Cells cultures were grown as described before using the *tnaA*::GFP strain. To estimate membrane potential in cells, a sample (1 ml or 0.5 mL) from a growing culture was removed, the OD_{600} measured and samples were frozen at -195 ºC by immersion in liquid nitrogen and then stored at -25 ºC until further analysis. When all samples were collected, an approximated volume of 10 µL was taken from each sample and placed onto a Agarose (1,5%) slide and pictures were taken under the microscope, Nikon ECLIPSE LV150L with an immersion oil objetive 100x NA 1.3. GFP signal was passed through a GFP cube filter (Nikon Corporation) and pitures were taken with a CCD camera DS-5Mc (Nikon Corporation). Images were analyzed with Fiji.

5.5 Data Analysis

All data was analyzed using R studio environment based on R 3.2.3. Growth regression models were calculated with the package GroFit[18] and graphics where created with Lattice and Lattice Extra[38]. Specific packages where used for flow cytometry data[23,37].

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