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E.coli splits into subpopulations exhibiting different membrane permeabilities upon switch to nutrient limiting conditions : To hedge the bet or not ?"

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Abstract

Gram negative bacteria are characterised by the presence of an outer membrane (OM). It has been shown that upon glucose limiting conditions the bacterial population splits into two subpopulations exhibiting low and high OM permeability respectively. Such phenomenon could be interpreted as a bet-hedging strategy, with the subpopulation expressing high OM permeability exhibiting higher nutrient uptake and the subpopulation with low OM permeability being more resistant to potential stressor.

In the first part of this work, a set of *E.coli* mutant strains displaying different ratio between OM permeabilized and non permeabilized subpopulations have been challenged for their growth fitness under nutrient limiting conditions. Mutants with higher OM permeabilized subpopulation, except the ones with OMPA deletion, exhibited increased fitness under limitation, suggesting a bet-hedging mechanism. However, deletion of OMPA resulted in a decreased fitness, probably due to the structural impact of this important porin. In a second stage, dynamics of the OM permeabilized subpopulation has been tracked based on on-line flow cytometry. A highly non-monotic dynamics was observed at this level, suggesting that several cellular mechanisms are implied in the adaptation to nutrient scarcity. More specifically, these results can be interpreted as a succession of two adaptive mechanisms, i.e. with the first one being fast and implying a fraction of cells with increased nutrient uptake capacity and the second one, slower, implying a significant reduction in cell size. As a conclusion, a bet-hedging mechanism has been observed during the first phase of adaptation to nutrient limiting conditions, this mechanism being then followed by deeper modification of the cellular structure. Our results point out the fact that the mechanisms of adaptation of a microbial population to nutrient limitation are complex and involved probably a cost-benefit effect leading to a succession of strategies.

Résumé

Les bactéries Gram - se distinguent par la présence d'une membrane externe. Il a été démontré que dans une situation de limitation en glucose, *E.coli* se sépare en deux sous-populations présentant respectivement une faible et une forte perméabilité de la membrane externe. Ce phénomène peut être interprété comme une stratégie de 'bet-hedging' (distribution des risques), la sous-population présentant une membrane externe plus perméable ayant une plus grande capacité d'absorption de nutriments et la sous-population présentant une faible perméabilité membranaire étant plus résistante à un potentiel stress.

Premièrement, la croissance en conditions de limitation en glucose d'un ensemble de mutants d'*E.coli* présentant des ratios différent du phénotype perméable a été évalué. Tous les mutants, à l'exception de ceux comprenant la suppression de OMPA, ont montré une meilleure croissance en conditions de limitation. Ceci semble confirmer la présence d'un mécanisme de bet-hedging mais l'évaluation de celui-ci a été complexifié par les mutations utilisées. En effet, probablement due à l'impact structurel de cette importante porine, la suppression de OMPA a entraîné une diminution de la croissance en conditions non-limitante en glucose. Dans un deuxième temps, la dynamique de la sous-population perméable a été suivie à l'aide d'une cytomètre en flux en ligne. Dès lors, il a été observé que plusieurs dynamiques étaient impliquées ce qui suggère que plusieurs mécanismes cellulaires entrent en jeux dans l'adaptation au manque de glucose. Plus spécifiquement, ces résultats peuvent être interprétés comme une succession de deux mécanismes adaptatifs, le premier intervient rapidement et mène à une fraction de cellules ayant une capacité accrue d'absorption des nutriments et le second, plus lent, implique une réduction significative de la taille des cellules. En conclusion, un mécanisme de bet-hedging a été observé durant la première phase d'adaptation à la limitation en glucose, ce mécanisme a ensuite été suivi par une modification plus approfondi de la structure cellulaire. Nos résultats soulignent le fait que les mécanismes d'adaptation d'une population microbienne à la limitation en glucose sont complexes et impliquent probablement une balance bénéfice-coût conduisant à une succession de stratégies.

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1 Context

The past few decades has seen the appearance of technological innovations such as flow-cytometry, microfluidics and microarray DNA analysis that have allowed single cell analysis. With a better awareness of cell to cell heterogeneity, microbiology has been shifting from population scale characterisation to single cell analysis[Brehm-stecher and Johnson, 2004]. Such single cell analysis has revealed that even in homogeneous environmental conditions, an isogenic population exhibits heterogeneity in gene expression [Natarajan and Srienc, 2000] [Balaban et al., 2004][Hua et al., 2004]. This variability in gene expression represents a challenge to the stability of industrial fermentation [Delvigne and Goffin, 2014], can lead to the appearance of antibiotic resistance [Balaban et al., 2004] and the switch to pathogenic behaviours [Weigel and Dersch, 2018].
Phenotypic heterogeneity is attributed to stochasticity, also called noise, in gene expression and can lead to the formation of multiple sub-populations displaying different behaviours. Such heterogeneity is not without interest and can benefit the overall fitness of the population trough risk-spreading. Also known as bet-hedging, this strategy encourages diversity because it increases the probability that one of the offspring will be more adapted to new challenging conditions [Schrott et al., 2009]. Thus, the degree of heterogeneity exhibited by a clonal population can be amplified by stressful environmental conditions [Schreiber and Ackermann, 2020].

Escherichia coli belongs to the *Enterobacteriaceae* family and is known because of its potential pathogenic capabilities as well as its industrial importance. The bacteria is present in the intestinal tract of warm blooded animals but can also thrive in totally different habitats such as lake water [Ackermann, 2015]. As a Gram - bacteria, *E.coli* has a double membrane and then, is characterised by the presence of an outer membrane that acts as the first filter and barrier to exchanges with the environment. Since it can be found in a wide diversity of environments, *E.coli* is susceptible to encounter different osmotic conditions, nutrient access and toxic compound exposure [Darcan, 2012]. This diversity of habitat has led the bacteria to develop a sophisticate machinery composed of outer membrane proteins called porins and a regulation network that senses and responses to the surrounding environment through specific porins production and abundance managing [Smith et al., 2007][Liu and Ferenci, 1998][Dam et al., 2018]. There is a wide diversity of porins, each having a different size of pores. As often a trade-off is associated with the choice of a certain porins combination by a bacteria. Since porins edict outer membrane permeability, the bacteria has to carefully sense its environment to maximise nutrient uptake, while minimising the risk of taking in harmful substances such as bile or antibiotics.

E.coli transcriptional analysis revealed that 9.8 % of 4,071 studied genes were impacted when growing in glucose limiting conditions [Hua et al., 2004]. It unveils that glucose limitation acts as a trigger for phenotypic diversity. One of the many characteristics influenced by glucose deprivation is the outer membrane permeability [Dam et al., 2018]. Previous results showed that glucose limiting conditions leads to the appearance of a permeable sub-population [Sassi et al., 2019]. Such population split are not un-heard of and can be explained by the fitness gain associated to having multiple and diverse sub-populations. This is known as bet-hedging and translates into ameliorating the overall fitness of the population by promoting multiple sub-populations, thus increasing the probability that one of them will be adapted to new conditions[Ackermann, 2015].

A previous screen of porins knockout mutants of the keio library revealed that the deletion of certain porins increases the abundance of a sub-population with a high outer membrane permeability. Prior to this work, *E.coli* W3110 Δ*ompC* was grown in glucose limiting conditions while pulsing glucose to encourage phenotypic diversity. In light of these results and with the additions of experimental observation of phenotypic diversification of other porin deleted mutants, this work ambitions to address the dynamics of permeabilisation, the role of porins in this process and how the observed bistability increases or not the fitness of the population. Flow cytometry was used to acquire data at a single cell level. A comprehensive understanding of the outer membrane permeability is of great interest for industrial process stability, environmental and bio-medical application.

2 Isogenic cells in a stressful environment can exhibit different phenotypes as a bet hedging strategy

An isogenic population in a homogeneous environment experiences diversification either as a result of the accumulation of mutations (genotypic diversity) or because of cell to cell variation in gene expression from a clonal population (phenotypic diversity) [Martins and Locke, 2015].
Because of the relatively short time scales of biotechnological processes, genotypic diversity is not the main driver of diversification thus phenotypic heterogeneity is considered to be a major source of variability [Delvigne and Goffin, 2014]. Although phenotypic diversity can result from a fluctuating environment [Thattai and Van Oudenaarden, 2004], a clonal population exhibits phenotypic heterogeneity even in homogeneous environmental conditions.

2.1 Phenotypic diversity arises from biological noise

Part of this diversity can be explained by stochasticity in gene expression, also referred to as biological noise. This noise can be split into two major origins, intrinsic and extrinsic.

Gene expression is complex and depends on multiple macro-molecules such as polymerase, DNA and regulatory proteins. These components also derive from composite reactions, generation variations in their availability and concentration. Since gene expression is tied to this varying supply of components, the output of the gene itself is fluctuating [Swain et al., 2002]. It is referred to as extrinsic noise. But it has been shown that even with identical intracellular environment, gene expression is still noisy [Elowitz et al., 2002]. This inherent noise, called intrinsic noise, derives from the stochasticity associated to the reunion of elements needed for gene expression. Indeed, 80% of *E.coli* genes are expressed to less than a hundred copies inside of a crowded intracellular environment [Ellis, 2001]. As a result of this, a probability is associated to the gathering of these rare and necessary elements to gene expression at the right time and suitable concentration.
While noisy gene expression is at the root of phenotypic heterogeneity, gene architecture itself can encourage diversification [Koh and Dunlop, 2012]. Acting together to induce sub-populations, they increase the fitness of the population by providing offspring ready to adapt to a new environment [Veening et al., 2008].

2.2 Gene circuit architecture can lead to bistability

Gene expression is controlled by one or more transcription factors that are activated or produced in the cell depending on environmental conditions. The regulation network of a gene is made out of all the transcription factors and other genes that control its pattern

of expression. These component can be tied through positive and or negative feedback loops. Network architecture can then promote or not noise, thus since the gene output is under selective pressure, network architecture and its degree of noisiness is also prone to selective pressure [Koh and Dunlop, 2012].

According to the theory of bet-hedging, having a noisy expression can be advantageous. Depending on the type of network architecture, a population can exhibit two stable phenotypic states [Dubnau and Losick, 2006]. Such a sharp difference can be explained by comparing the production and degradation rate of a potential regulatory protein (figure 1). If the protein production is under the control of a specific regulatory mechanism, its production profile is following a Hill equation and follows a sigmoïdal curve in function of the abundance of the protein itself. Such pattern is found for example in the case of a simple positive autoregulatory motif, where the protein activates its own production [Alon, 2019]. When comparing the concentration profiles for the rate of protein production and degradation, three nodes can be observed. It can be demonstrated that the two extreme node corresponds to two stable steady state. At the population level, these two steady states are expressed simultaneously by two subsets or subpopulations of cells, leading to bistability.

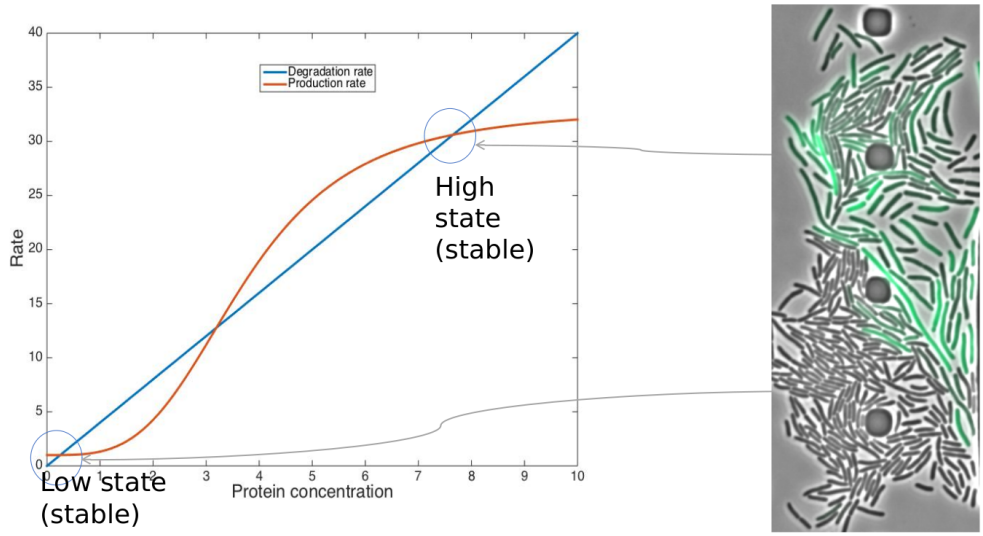

Figure 1: Difference in production and degradation rate of a protein can lead to bistability

This mathematical interpretation of bistability is at the basis of the generation of bet-hedging strategies because potentially leading to the the appearance of two subpopulations with different adaptive capabilities. But not all strategies have the same cost, nor do they yield identical benefit. It was shown that noise in gene expression is minimised if variation in this gene can be lethal [Fraser et al., 2004]. Then, the ratio of cells switching from one gene expression state to another can be predicted by taking into account the benefit cost associated to one gene expression state as well as the probability of fluctuation in the environment [Thattai and Van Oudenaarden, 2004].

2.3 Segregostat: a continuous cultivation device that senses and reacts to phenotypic diversity

Segregostat is the name given to a novel type of reactor that allows for the characterisation of the dynamic time scales related to population diversification and the conditions for stabilising subpopulations. The device is composed of a flow cytometer that quantifies the degree of diversification of the microbial population under cultivation. Then, the apparatus controls the population heterogeneity based on nutrient pulses, i.e. most of the time a carbon source.
Because stimulus pulses have been shown to allow gene expression control [Benzinger and Khammash, 2018], the notion of pulse is at the core of the segregostat. As previously described, glucose limitation acts as a strong trigger for phenotypic diversification. By pulsing glucose if the proportion of the permeable phenotype went bellow a 10% threshold, this device has successfully maintained *E.coli* BW25113 Δ*ompC* permeable phenotype fraction at 10% during 40 hours in glucose limiting chemostat cultivation[Sassi et al., 2019].

While the segregostat has proven its ability to control phenotypic diversity, interrogations concerning the dynamics of the phenomena as well as the role of porin removal in it remains.

3 Single cell proxies and devices for evaluating outer membrane integrity and permeability of Gram-negative bacteria

3.1 Propidium iodide: a practical but poorly understood dye for outer membrane permeability assessment

Propidium iodide (PI) is a DNA and RNA intercalant dye with little or no sequence preference. When attached to DNA or RNA, its fluorescence intensity increases 30 fold and excitation and emissions maxima shift respectively to 535 nm and 617 nm.
Because PI stoichiometrically binds to nucleic acids, it is utilised to determine cell DNA content [Riccardi and Nicoletti, 2006]. Since dead bacteria fail at keeping propidium iodide outside of the cell, apoptosis can be monitored through PI staining This is done using the channel FL3-A of flow cytometry that enables single cell and quantitative analysis of PI staining [Riccardi and Nicoletti, 2006]. Bacteria below a fluorescence value of 10^3 are considered unstained and thus have an intact, none permeable outer membrane. Fluorescence value above 10^5 is indicative of cell death.
But upon glucose limitation, a partially stained sub-population with fluorescence values close to 10^4 is present [Sassi et al., 2019].

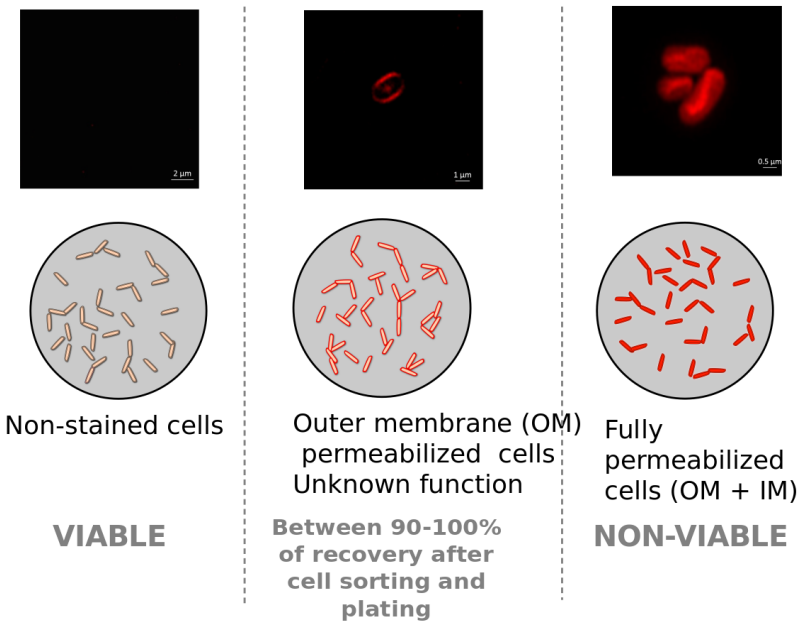

Figure 2: Different observed pattern of PI staining [Sassi et al., 2019]
Partially stained cells are viable bacteria stained in the area of the outer membrane by an unknown mechanism

Confocal microscopy (figure 2) has revealed that this partially stained population emits fluorescence somewhere in the area of the outer membrane and of the periplasm but not in the cytoplasm as dead bacteria would normally do [Sassi et al., 2019]. The reason behind this partial staining is unknown but the phenomena is not un-heard off. Since

nucleic acids can be found outside the microorganism, propidium iodide tends to over-evaluate cell death.
Extracellular DNA (eDNA) is involved in cell attachment and biofilm maturation. It has been observed that, both for *Staphylococcus epidermidis* and *E.coli*, apoptosis is over-estimated in the context of cell surface attachment [Rosenberg et al., 2019]. Even if eDNA involvement in false positive PI staining has not been proven for *E.coli*, it represents a plausible hypothesis. Membrane stressed *Saccharomyces cerevisiae* have also been shown to be prone to PI staining while being alive (RBND read but not dead) [Davey and Hexley, 2011]

Another hint on why PI is capable of producing false dead signals could be found in its over usages (figure 3). Unlike microbiologists that use PI as a dye specific to nucleic acids, plant physiologist have long been using it as a staining agent for plant cell walls (figure 3).

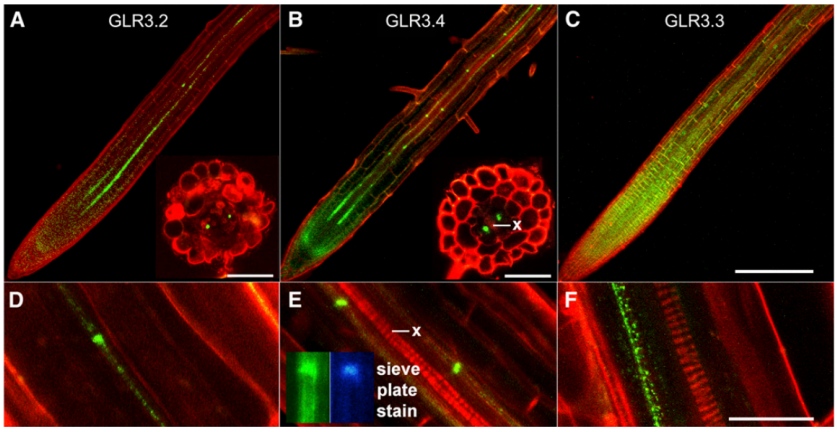

Figure 3: Plant cell walls stained by propidium iodide [Vincill et al., 2013]

It has been proposed that in the context of plant cell wall, and more specifically for pollen tubes and arabidopsis root hairs, PI competes with Ca^{2+} and binds to pectin [Rounds et al., 2011]. Addition of calcium ions decreases the staining, showing the competition between PI and Ca^{2+} ions. Alternatively, the alteration of cell wall through pectin methylesterase increased the staining.

Such PI binding to non nucleic acid compounds can be explained because of a localised deficit in p-orbital electrons at the level of four nitrogen atoms making PI partially positive. This allows interactions with negatively charged hydroxyl and carboxyl groups of the galacturonic acids that constitutes pectin. Both examples of propidium iodide staining confirms that this dye is more than a cell death proxy. Understanding exactly why this dye is exposes this particular sub-population would definitely help characterising it.

3.2 1-N-phenylnaphthylamine: known mode of action but impractical for single cell analysis

The use of 1-N-phenylnaphthylamine (NPN) is another way to analyse outer membrane permeability. If damaged or permeabilized, this hydrophobic compound passes though the outer membrane into the periplasm. When passing from an aqueous to a hydrophobic environment, NPN fluorescence increases sharply.
Since this dye acts in a different way than PI, it can be used to confirm that PI properly enables the monitoring of outer membrane permeability. But having an appropriate dye is not enough for single cell analysis, it also has to be compatible with equipment allowing its detection at a single cell level. Sadly, NPN cannot be used with flow cytometry since none of the usual fluorescence monitoring channels are compatible with the excitation and emission wavelength of NPN.

3.3 Flow cytometry for high-throughput, single cell, analysis

Flow cytometry (FC) is one of the powerful instruments that has enabled the development of single cell analysis. A bacterial suspension is diluted in an isotonic fluid and then pumped as a laminar flowing stream. The stream passes by a beam of monochromatic light and each particle, called event, is analysed [Brown and Wittwer, 2000].

Light scattering provides morphological information on the event. Forward scatter (FSC) is the diffraction in the beam axis and is linked to size event. The SSC channel, side scatters, measures the reflected light at a $90°$c angle. This gives information on the 'internal complexity' of the event. Even though the understanding of this notion is not as intuitive as the event size, the combining of the two helps to distinguish the morphological characteristics of the sample.

Fluorescence can also be monitored at a single cell level through FC. The use of a specific dye, tagged genes and fluorescent label means that flow cytometry also offers the possibility to observe gene expression, DNA content or any imaginable characteristics that can be linked to fluorescence.

3.4 Microfluidics for single cell analysis under stable environmental conditions

Microfluidics are small chips made out of a transparent bio-compatible material such as PDMS (polydimethylsiloxane) [Wlodkowic and Darzynkiewicz, 2011]. These chips are separated in multiple picoliter bioreactors (PLBRs) that are composed of a network of microchannels (1 to 1000 μm) associated to cultivation chambers (figure 4). Such small volumes means that fluid flow is laminar and then the conditions inside the cultivation chambers are homogeneous.

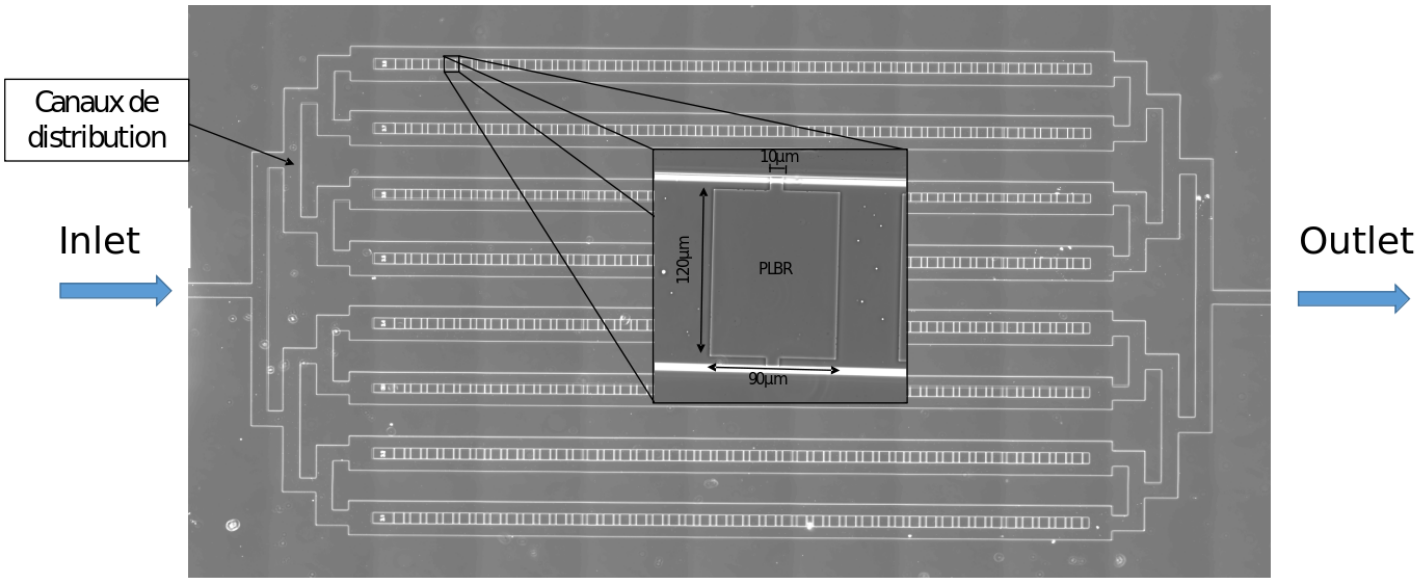

Figure 4: One PLBR contains an inlet and an outlet that splits into multiple channels feeding cultivation chambers

Microfluidics also benefit from the versatility of their design to meet the requirement of the experiment [Grünberger et al., 2014]. Thus, to observe both growth and morphology, the chip used in this work allowed 2D growth to maximise the quality of the observation, while providing a good perfusion to avoid any formation of a glucose gradient. Sadly since the impact of PI addition during cultivation on cell is unknown, growth and morphology could not be observed at the same time as outer membrane permeability. Beside, the real concentration of PI inside of the microfluidic cultivation would be unknown due to possible absorption of the dye on the walls of the device (Alexander Grünberger, personal communication).

4 Porin dynamics and population fitness

4.1 Porins are the bridge between the inside of the cell and its environment

Additionally to the usual inner membrane (cytoplasmic membrane), Gram - bacteria are characterised (figure 5) by the presence of an outer membrane (periplasmic membrane). The inner membrane is made out of a bi-layer of phospholipids and then is described as symmetrical. On the contrary, because the composition of the inner and outer leaflet is different, the outer membrane (OM) is asymmetrical.

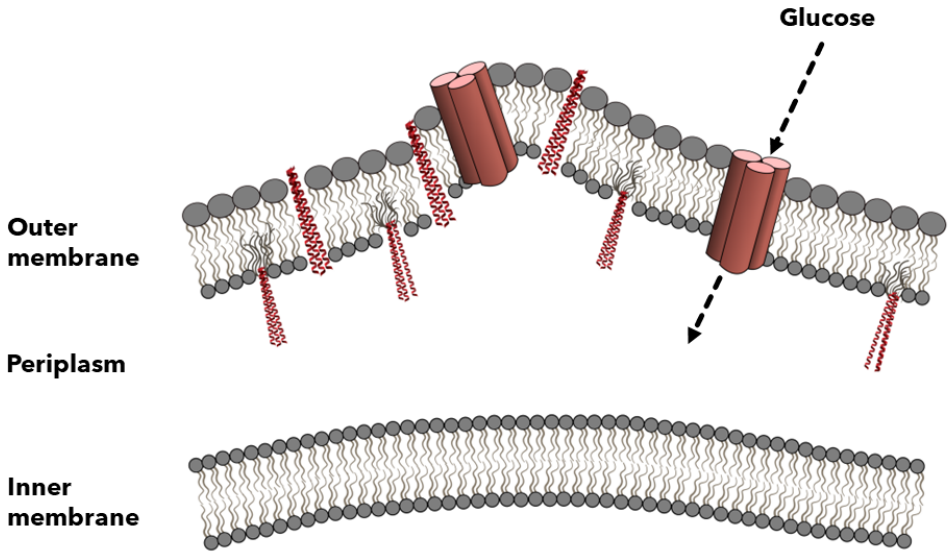

Figure 5: Representation of the inner membrane and outer membrane with LPS and embedded porins allowing the passage of glucose.

Separating the inner and the outer membranes, the periplasm is filled with peptidoglycan named mureins. This rather homogeneous layer gives strength to the cell and is attached to proteins of the outer membrane [Quintela et al., 1995]. The mureins layers act as a net that is both slightly deformable and permeable since it allows the passage of globular proteins up to 100 kDa [Vollmer and Bertsche, 2008]. Similarly to the cytoplasmic membrane, the inner leaflet of the outer membrane is made out of phospholipids. The outer leaflet is a complex mixture of lipopolysaccharides (LPS), phospholipids and membrane proteins. The LPS present in the outer limits of cell are recognised by our innate immune system and are also known under the name of endotoxins [Ulevitch and Tobias, 1999]. The phospholipids and lipopolysaccharides that compose the outer membrane act respectively as barrier against hydrophilic and lipophilic molecules thus isolating the bacteria from its surrounding environment [Kamio and Nikaido, 1976].

Being a living organism implies the aptitude of exchange with its surrounding environment. To do so, Gram - utilise β-barrel shaped membrane proteins. These pores

forming proteins (porins) render the outer membrane more leaky than the cytoplasmic one [Koebnik et al., 2000].

Many porins have been identified and while some of them are extremely abundant, many more are rare. Since each of these porins can be distinguished by their affinity with less of more specific solutes, porin balance depends on external conditions [Viveiros et al., 2007] [Dam et al., 2018]. As often, a trade-off is associated to the choice of a combination of porins. Larger pores forming one allow for a better influx of nutrient but also expose the cell to detrimental osmotic conditions and/or the presence of toxic compounds. Small porins makes bacteria more resistant but less capable of taking in essential building blocks.

A classical example of this tarde-off is how *E.coli* adapts the abundance of outer membrane proteins A and F (OMPA and OMPF) depending on environmental conditions. *E.coli* being present in guts and lake water, the feacal bacteria has to adapt to diverse conditions [Özkanca and Flint, 2002]. Nutritionally speaking, guts are a rich environment but many toxic compounds such as bile are also present in large amounts. Also, osmotic conditions are not favourable.
By contrast, lake water is a fresh and poor environment with low osmolarity. Nutrient are scarce but so are toxic substances. OMPA is the most abundant porin and is considered to form small pores compared with the less present but bigger pore formating OMPF. Unsurprisingly, OMPF relative abundance to OMPA increases greatly in lake water. Contrariwise, OMPF is far less abundant when bacteria are growing in guts. This is a great example of the trade off associated to outer membrane composition. Allowing large pores in the OM is primordial for nutrient access but it is this very same solution that makes it susceptible to harmful compounds such as bile or antibiotics.

The choice of porins to be suppressed in this work was done based on a previous screen of the Keio library (Frank Delvigne, unpublished results). The porins OMPA, OMPC and LamB were chosen since their deletion has an impact on the overall outer membrane permeability of *E.coli*.
Outer membrane proteins regulation happens at transcriptional, pre-traductional and post-traductional levels. The regulations as well as the characteristics of some of the most abundant porins including the ones that were deleted in this work will be presented in the following sections.

4.2 OMPA: A major porin with a structural role

Encoded by *ompA* gene, outer membrane protein A is one of the most abundant porins. As all other porins, this 325 amino acids long protein is characterised by a two domain structure. The N terminal domain is a β-barrel located in the outer membrane [Smith et al., 2007] and the C terminal section is located in the periplasm.

Unlike other porins, for a long time it has been unclear if the channel formed by the β-barrel was open or not [Sugawara and Nikaido, 1992] [Sugawara and Nikaido, 1994]. It was discovered that only a small proportion of OMPA (2-3%) contained an open channel.

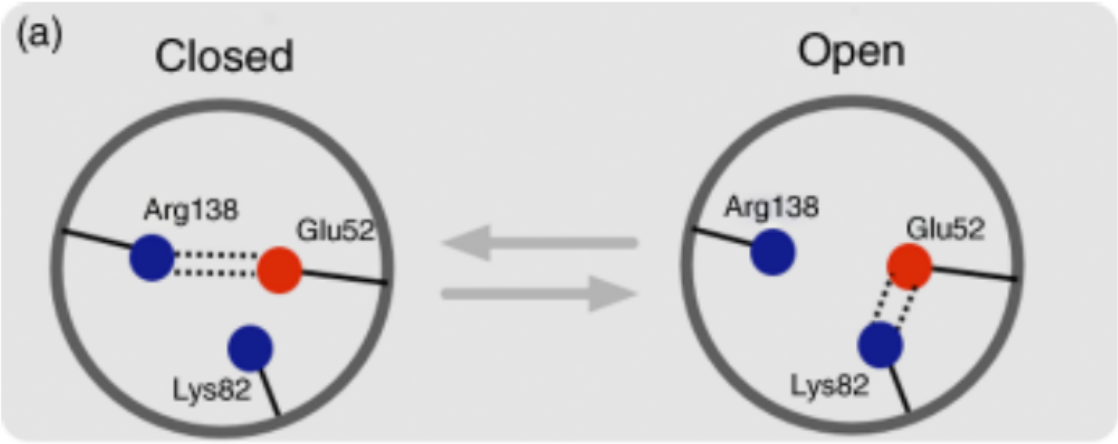

Figure 6: Illustration from [Smith et al., 2007] showing the gating mechanism of OMPA channel

The 2007 review from Smith et al. offers a detailed description of this complex structure [Smith et al., 2007]. As seen on figure 6, the channel is open or close depending on salt bridge arrangements. Also, the usual 8-stranded β-barrel can become a 16 stranded one. This irreversible change leads to a larger pore and is temperature dependant. According to Zakharian et al. , it occurs at temperatures of or higher than $26°$c. During this enlargement, all amino acids from the c-terminal domain that are normally present in the periplasm change of domain and are included into the β-barrel. The size of this last passing from 171 to 325 residues [Zakharian and Reusch, 2005].

Since OMPA is tightly associated with the peptidoglycan layers, this porin also has a structural role [Wang, 2002]. This structural role means that the deletion of *ompA* as well as the *llp* gene, responsible for murein lipoprotein, leads to a spherical bacteria [Sonntag et al., 1978]. This unusual shape as well as the floating peptidoglycan layer can be observed on this remarkable electronic microscopy image from 1978 (figure 7).

This OMP being so present, it also represents a target. OMPA is a receptor for bacteriocins such as colicin, bacteriophages and even some components of the immune system [Kim et al., 2014] [Smith et al., 2007].

4.2.1 *ompA* regulation

The production of OMPA is tightly regulated and depends on environmental conditions. Mechanisms that modulates the abundance of OMPA happen at different levels but, the transcript stability one is well described. Because of its folded 5' extremity, *ompA* mRNA

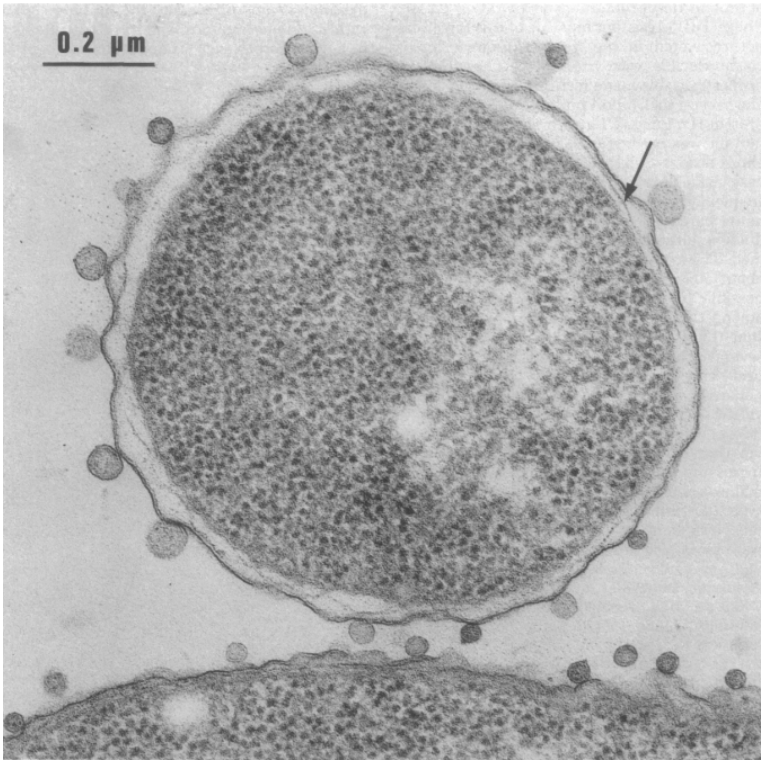

Figure 7: Electronic microscopy image of *E.coli* mutant lacking *ompA* and murein lipoprotein from [Sonntag et al., 1978] showing the spherical shape of the resulting bacteria and the free floating peptidoglycan layer

is stable. By tuning the turn-over of *ompA* mRNA, the abundance of the porin can be changed [Smith et al., 2007]. Passing from roughly 15 mins at exponential phase to 4 mins at slower growth rate, *ompA* mRNA half life increases with growth rate. At low temperature and growth rate, Hfq protein destabilises the *ompA* transcript by attaching itself to the SS2 sequence, thus unfolding the 5 ' end. The protein also alters the capabilities of ribosomes to bind the *ompA* mRNA. It does so by stabilising the binding of the small RNA MicA to the ribosome binding site (RBS) of the transcript.

MicA sRNA is positively regulated by the *rpoe* gene and its transcription factor, the envelope stress sensor *sigma E*[Udekwu and Wagner, 2007] [Guillier et al., 2006]. Under favourable conditions, *rpoe* is not activated and the stable *ompA* mRNA is traducted. But envelope stress, of which a too great abundance of OMPA, trigger the expression of rpoe through the transcription factor *sigma E*. Then with the help of Hfq, MicA binds to *ompA* transcript and degrades it. But *sigma E* factor activity can also be increased by other factors than extracytoplasmic stress. The *sigma E* factor can also be up-regulated by nutritional stress signal such as ppGpp [Costanzo and Ades, 2006] and thus, starvation represses OMPA . This is in line with the observation that *ompA* mRNA half-live is at least three fold smaller during low growth rate phases.

Another actor of the post transcriptional regulation of *ompA* is the exoribonuclease RNase R. *rnr* knockout mutant was shown to contain more OMPA during stationary phase than the wild type [Andrade et al., 2006]. This indicates that *ompA* mRNA is degraded by RNase R during this phase.

4.3 OMPC and OMPF are antagonistically expressed

The outer membrane protein C is the third most abundant porin. It allows in smaller and more cationic solutes than OMPF. These two are described together since their regulation is linked [Liu and Ferenci, 2001]. The two OMP's encounter for 2 % of all porins, making them major contributors to membrane permeability management.

Both exhibit the β-barrel shaped pore specific to porins. They are identical at 60 % but unlike previously thought, they have similar pore sizes. The reason behind the greater permeability of OMPF is likely due to differences in the pore electrostatic environment [Baslé et al., 2006]. The relative abundance of these porins depends on the availability of nutrients, osmolarity and temperature. The ratio OMPF/OMPC increases when nutrient are scarce and osmolarity is low. The two porins respond to the same stimulus but in an antagonistic way. As an example, although the way the glucose shortage messenger cyclic adenosine monophosphate (cAMP) interacts with the promoters of *ompC* and *ompF* is unknown, it was shown that its presence up-regulates *ompF* while decreasing *ompC* [Liu and Ferenci, 2001]. *ompC* and *ompF* transcriptions are under the control of multiple distinct genetic circuits. Some of the main ones and most described will be developed bellow.

4.3.1 OMPR-EnvZ

The double component regulator OMPR-EnvZ manages the two porins abundances in regards to osmotic conditions [Batchelor et al., 2005]. OMPR, under the control of *ompr* gene is phosphorylated by EnvZ. The newly produced OMPR-P then binds up-stream of *ompC* and ompF to either promote or inhibit their expression.
Where a low level of OMPR-P promotes the transcription of *ompF*, a high level inhibits *ompF* and promotes *ompC*. EnvZ presence increases with high osmotic conditions, but it is unclear if other factors such as glucose limitation impact the overall abundance of the kinase [Özkanca and Flint, 2002]. The histidine kinase Env-Z is described as an osmosensor but the way osmolarity triggers its activation remains unknown.

4.3.2 CpxA-CpxR

Another dual component regulation system is CpxA-CpxR. Envelope stress such as alkaline pH or overexpression of porins releases CpxA that activates CpxR by phosphorylating it [Batchelor et al., 2005]. The activation of the second component strongly decreases *ompF* expression and increases *ompC* one. Strangely enough, *sigma E* that also is an envelope stress sensor does not have the same effect than the dual system CpxA-CpxR. Where the first reacts to membrane stresses by increasing the production of OMPC and decreasing OMPF, *sigma E* inhibits the production of both. It has been hypothesised that the system CpxA-CpxR is more sensitive to toxins than *sigma E*, allowing the cell

to adapt by replacing the large porin OMPF by OMPC instead of repressing both of them and isolating the cell [Batchelor et al., 2005].

4.3.3 Mic C and Mic F

Small RNA acts at a post-transcriptional level by binding to mRNA. By doing so, it destabilises it or blocks the ribosome binding site (RBS) thus acting as a riboregulator.
micF and *micC* respectively encode the small RNA MicF and MicC [Chen et al., 2004]. Toxins exposure and high temperature activates *micF* that decreases *ompF* traduction. Similarly to *micF*, *micC* binds to *ompC* mRNA RBS leading to a decrease in OMPC production. The small RNA MicC is most present in colder conditions, when glycerol is used as a carbon source or when cells are exposed to osmotic shock.

All in one, the regulation of OMPF and OMPC converges and makes sense in light of the notion of fitness. Cells have to balance growth and resistance to ensure gene propagation. Even though the regulation circuits are abundantly described and studied, much remains unknown. By instances, it has been observed that glucose limitation increases the presence of OMPF but the mechanism by which it does so is not clearly identified. The role of OMPR-EnvZ is supposed but not universally validated. [Liu and Ferenci, 2001]. cAMP also plays a role in the upregulation of *ompF* but the mechanism by which the glucose stress signal does so remains unknown [Liu and Ferenci, 2001].

4.4 The high affinity porin LamB

Compared to other above described porins, LamB is far less abundant. Its other name, maltoporin, is miss-leading for the readers that assumes this porin is exclusively used for taking in maltose were it allows the intake of all carbohydrate in limiting conditions [Death et al., 1993]. LamB contributes to growth in carbohydrate limiting conditions and its deletion does not have any significant impact on growth when glucose concentration is above 0.2 mM environment [Death et al., 1993]. Then, the role of OMPF and LamB are not redundant since their relative utility depends on the residual sugar content. As off *ompF*, *LamB* expression increases with glucose limitation. By binding on *LamB* promoter, cAMP receptor protein (CRP) enhances *LamB* expression [Vidal-Ingigliardi and Raibaud, 1991].

4.5 Porin turnover and organisation

To ensure good adaptation of the bacteria to its new environment, porin additions happens in parallel to porin removal. New porins tends to get more often included in the cylindrical portion of the rod shape cell than at its poles [Rassam et al., 2015]. Thus while bacteria are growing, outer membrane proteins are pushed away from the center to the polar region. This generates a phenomenon of memory, where as cell divide, daughter cells keep a certain part of the porins from the mother cell [Ursell et al., 2012].

The mobility of porins is mostly driven by new OM addition and not diffusion. Then, the composition of the outer membrane is renewed by dilution. As older porins are found at the poles and that each daughter cells inherit from one of them, two generations are needed to obtain a bacteria with completely new OMP's [Kleanthous et al., 2015]. Alongside the renewal of the outer membrane through dilution, parts of the outer membrane can be expelled through vesicles. Outer membrane vesicles are spherical particles of 20 to 250 nm composed LPS, OMP's, membrane lipids and DNA [Kulp and Kuehn, 2010]. Beside from the above cited components, they can also transport signalling molecules, toxins and everything that is either too much or harmful for the cell [Lee et al., 2007].

5 Material and method

5.1 Strains, cultivation medium and preculture

The set of strains utilised in this study is composed of industrial strain *Escherichia coli* W3110 wild type and four porin lacking mutants, *E.coli* Δ*ompA*, Δ*ompC*, Δ*LamB* and Δ*ompA*:Δ*ompC* , generated through CRISPR cas 9 deletions by Syngulon.
All precultures and cultivations were performed in minimal media (M9) containing in (g/l): K_2HPO_4 14.6, $NaH_2PO_4.2H_2O$ 3.6; Na_2SO_4 2; $(NH_4)_2SO_4$ 2.47, NH_4Cl 0.5, $(NH_4)_2$-H-citrate 1. The autoclaved growth medium was then supplemented with 10 ml of a filtration sterilised (0.2 μm) mineral trace and thiamine solution made out of (3/9 trace solution, 3/9 EDTA 20.1 g/l, 2/9 $MgSO_4$ 120 g/l, 3/9 $FeCl_3.6H_2O$ 120 g/l, thiamine 1 g/l). The trace solution is composed of (in g/l): $CaCl_2.2H_2O$ 0.74, $ZnSO_4.7H_2O$ 0.18, $MnSO_4.H_20$ 0.1, $CuSO_4.5H_2O$ 0.1, $CoSO_4.7H_2O$ 0.21. A final glucose concentration of 5 g/l was used for all precultures, chemostat experiments and non glucose limiting microfluidics whereas a concentration of 10 mg/l was chosen for the glucose limiting microfluidics. Precultures were grown in 1 l baffled flasks containing 100 ml of culture medium inoculated with 0.5 ml of a bacterial glycerol stock solution (25% glycerol, $-80°$c) and grown overnight whilst shaking at 150 rpm $37°$c.

Glucose concentration used for the limiting conditions was selected to induce nutrient stress. It has been described that a glucose concentration of 10^-6 M (1.8 mg/l) induced high glucose stress response [Notley and Ferenci, 1996]. Trials with 2.5 and 25 mg/l respectively resulted in no growth and in unaltered growth. Then, a concentration of 10 mg/l was successfully tested and selected.

5.2 Chemostat cultivation

To evaluate the impact of porin deletion on the appearance and the dynamics of the permeable phenotype, the wild type and each porin lacking mutants were grown in chemostat conditions during fifty hours. Samples were taken every ten hours to assess the ratio of the permeable phenotype.
Each strain was grown in triplicate using disposable stirred-tank milliliter bioreactor (bioReactor 8, 2mag München-Germany) with a working volume of 16 ml. Aerobic cultivation took place at $37°$c with an agitation of 2600 rpm. Inside the milliliter bioreactor, 10 ml of M9 media with antifoam 911 (2 drops par litre) was inoculated at an OD_{600} of 0.1 from an overnight preculture. Using a fluorimetric sensor of dissolved oxygen (DO) located at the bottom of the disposable reactor, the DO was monitored to observe the drop in dissolved oxygen level indicating the start of chemostat conditions. Then a set of two peristaltic pumps (Watson marlow 323) constantly added fresh medium and removed cultivation medium with identical flow (figure 8).

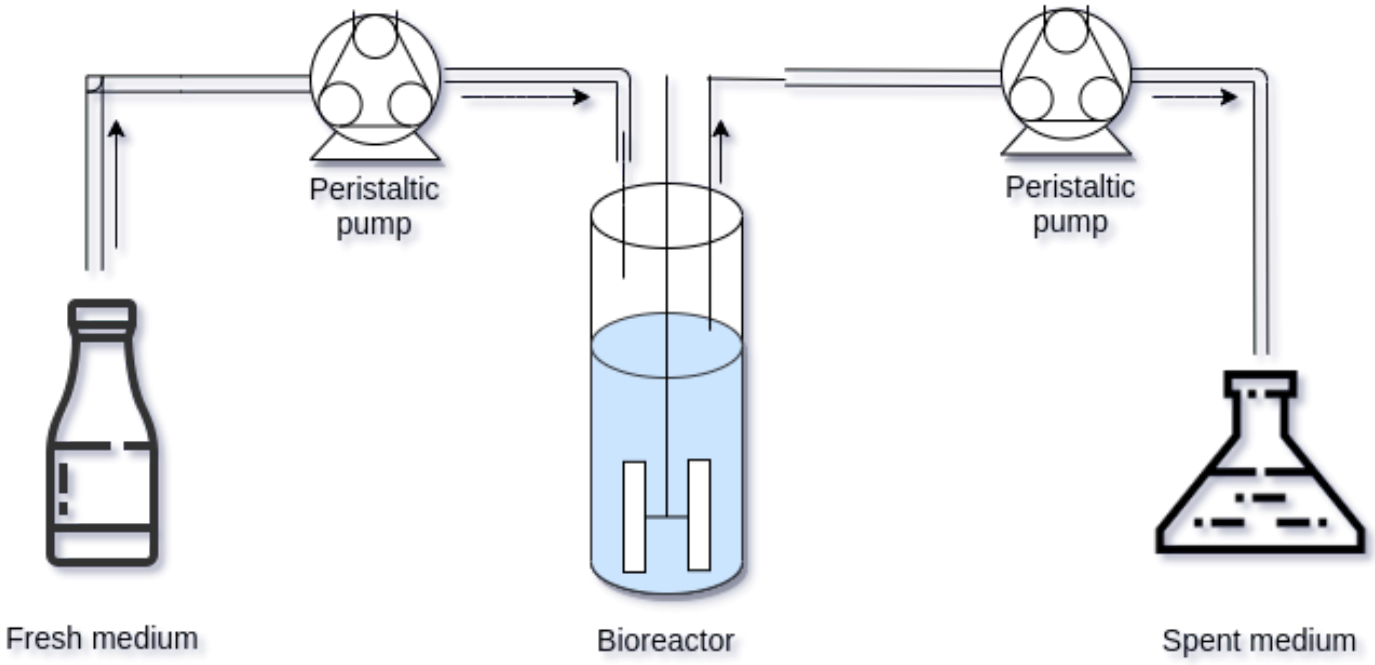

Figure 8: Representation of the 2 mag chemostat device assembly
Cultivation media is continuously pumped in and out of the bioreactor to achieve a dilution rate of 0.1 h^{-1}

Fresh medium is pumped from a Schott bottle through a tube (watson marlow Marprene Manifold HS 978.0025.00+) connected to a 50 mm long needle. The needle was placed inside of the cultivation device while the other extremity was plunged in a 1 l Schott bottle filed with M9 5g/l glucose.
The outlet tube (watson marlow Marprene Manifold HS 978.0129.00+) is connected to a 70 mm needle. This needle is bent at a 90 degree angle 1 cm from the upper connection extremity. This allows the tip of the needle to reach the cultivation device at a height that corresponds to a 10 ml volume.
To achieve a dilution rate of 0.1 h^{-1} that leads to glucose limitation [Wick et al., 2002], the pump was set on 5 rpm. While the flow of fresh medium is precise, the outlet flow capacity is higher. But since the needle extremity is at the liquid/air interface, the used cultivation medium removal matches fresh medium influx.

5.3 Continuous cultivation with glucose pulses

E.coli Δ*ompC* was cultivated in a stirred bioreactor (Biostat B-Twin, Sartorius) in continuous conditions while pulsing glucose (100 mg/l per pulse) at a frequency of one pulse per hour. Data acquisition was performed every 12 mins via online FC analysis (figure 9).
Inside of the 2 l capacity bioreactor, a volume of 1l of M9 5g/l glucose was inoculated at an OD_{600} of 0.5 from an overnight preculture. The pH and temperature were respectively maintained at 6.9 and $37°$c. Stirring was set at 800 rpm with a 1 VVM aeration rate.

The drop in dissolved oxygen marked the depletion of oxygen and the start of continuous cultivation mode. Minimal M9 medium was fed without carbon source into the bioreactor at a dilution rate D = 0.1 h^{-1} . At an interval of 1 hour, a pulse of glucose was injected in order to increase as fast as possible (within 5 seconds) the global glucose concentration to 100 mg/L. The microbial population was tracked by an on-line flow cytometry device previously described (paper from Sassi). Briefly, each 12 minutes, a sample of 0.8 mL was taken from the bioreactor, automatically diluted and stained with PI and then injected into an Accuri C6 flow cytometer.

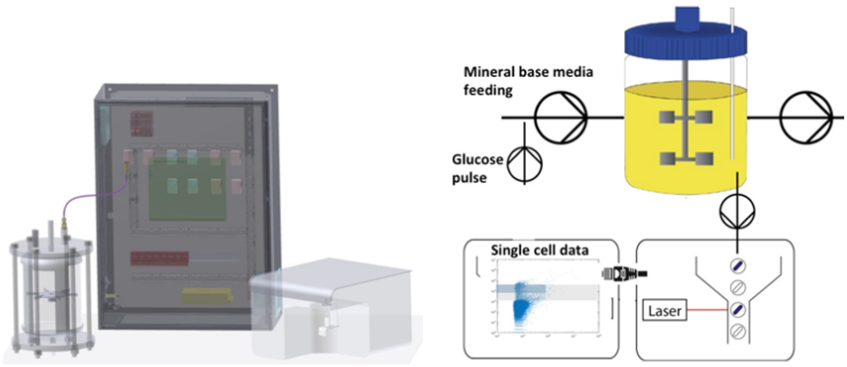

Figure 9: Online flow cytometry is used for analysing cell-to-cell differences (phenotypic heterogeneity) in membrane permeability following PI staining (adapted from [Sassi et al., 2019])

5.4 Outer membrane permeability assessment

To evaluate the proportion of the permeable phenotype, a sample was stained with propidium iodide (Invitrogen, Carlsbad US) before being analysed at a single cell level through flow cytometry

To avoid destabilising the chemostat cultivation, sample volume was limited to 20 µl. The sample was then diluted in PBS and propidium iodide (PI final concentration of 1 mg/l) before being left to incubate in the dark at room temperature during 10 minutes.

After staining, the sample was diluted with phospate-buffered saline (PBS) to fall in the 500-2500 events/µl range of the flow cytometer (FC). Using the FL3-A channel, propidium iodide fluorescence was analysed by a Accuri C6 FC (BD Accuri, San Jose CA USA). The analysis was performed with a medium flow (33 µl min-1) and a FSC-A threshold set at 20.000. The abundance of the permeable phenotype is defined as the ratio of partially stained cells (FL3-A value between 10 3 and 10 5) and the total number of analysed bacteria.

5.5 Microfluidic cultivation

Microfluidic cultivation associated to time laps microscopy was used to monitor growth, cell size and identify any morphological alteration of the four mutants and the wild type. Each of the five strains (mutants and wild type) were grown in microfluidic in glucose non limiting (5 g/l) and limiting (10 mg/l) conditions. All ten microfluidics experiments were identically prepared and conducted with differences only occurring between the cultivation in non limiting and limiting conditions.

The microfluidic chips used in this work were made out of the assembly of a PDMS chip (waffer 229 with a height of 778 nm) and a glass coverslip. The microfluidic is separated in picoliter bioreactors (PLBRs). Each PLBR has an inlet and an outlet that is previously formed using a hole puncher. Both of the components were washed before getting bonded together through plasma treatment. The PDMS was soaked in isopropanol and the coverslip in acetone for 30 seconds. Then the glass coverslip was washed with isopropanol and rinsed with miliQ water. The PDMS was washed in miliQ water. Both were then dried under a gentle flow of compressed air and bonded together after a 30 seconds plasma treatment. The assembly was left in an oven at $60°$c for half an hour to finish off the assembly.
Before any cultivation, the microfluidic chip was washed from the inside with a 4000 nl/min flow of M9 medium for 30 minutes. The flow passing in the PLBR was precisely adjusted with a press syringe (neMESYS low pressure syringe pump 290N). A one militer Omnifix®-F syringe was connected through tubing (Tygon Microbore tubing, 0.020" x 0.060"OD) to the PLBR inlet with an angled stainless steel tip. The medium outlet was assured by an other angled tip connected to a eppendorf by tubing.

To prevent cells from sticking to the PDMS, passivation was realised by flowing (1000 nl/min) a of bovine serum albumin solution (1 mg/ml in PBS) during one hour inside of the PLBR [Schrott et al., 2009]. Then the device was re-washed again with the medium that corresponded to the one later used for the cultivation itself.

After the microfluidic chip was assembled, washed, passivated and washed again, the cultivation began. The preculture was diluted 100 times in M9 containing either 5 of 10 mg/l of glucose before being flown through the PLBR to inoculate the cultivation chambers. When at least ten chambers had one cell inside, the PLBR was flushed with fresh medium containing the glucose concentration used for the experiment during two minutes (2 000 nl/min). This was done to ensure that the cultivation chambers and channels only contained medium with the correct concentration of glucose. The flushing also enabled the removal of unwanted cells present in the channels. Then using a Nikon Ti2 Eclipse reverse microscope with an oil immersion 100 X lens, images where taken every 15 minutes for ten hours. The chip was mounted on a plate heated at $37°$c and continuously fed with a 100 nl/min flow of M9 containing either 5 g/l or 10 mg/l of

glucose. Cell were manually content every hours and bacterial size over one division cycle was measured using the NIS-Elements Advanced Research software (Nikon, NY USA).

5.6 Evaluation of the bacterial glycogen content

Intracellular glycogen determination was performed to detect any difference in glycogen content of the strains at the end of the preculture. Precultures of same duration (15 hours) were realised for each stains. Then 20 ml of all precultures were centrifuged (9722 g for 10 mins at $4°$c) and washed three times with PBS to remove any residual glucose of the preculture from the pelleted cells.
The pellets were freeze dried and then suspended in HCl 0.9 M with a biomass concentration of 1 mg/ml. Intracellular glycogen was hydrolysed into glucose during five hours at $99°$c [Lanham et al., 2012]. The glucose was then analysed through HPLC (Agilent technology 1200 series) with a RID detector (HPLC method in annex 9.1). To get a technical triplicate, hydrolysis and analysis were performed three times. Because the yield of the glycogen hydrolysis is unknown, difference in glycogen accumulation was expressed as a ratio of the glucose peak area of the strain divided by the glucose peak area of the wild type.

5.7 Assessment of Dnase impact on propidium iodide partial staining

It was hypothesised that partial staining of *E.coli* came as a result of the presence of eDNA. The theory was tested by comparing the proportion of partially stained bacteria before and after Dnase treatment.
From a preculture of *E.coli* $\Delta ompA$:$\Delta ompC$, the number of cells was determine by FC and adjusted with PBS to around 10*7 cells/ml. Then, 1 ml of the solution was centrifuged (7 000 g for 10 mins) and the pellet was resuspended in 10x Dnase buffer. At this stage, the suspension was split in half, DNase I recombinant, RNase-free with a final concentration of 160 U/ml (Roche, Mannheim Germany) was introduced in one half while the other one was treated as a blank to evaluate if the Dnase buffer itself had any impact on the PI staining. Both the blanks and the Dnase added samples were left to incubate at $37°$c during two hours before being stained with PI and analysed through FC.

6 Results

6.1 Porin deletion exacerbates the abundance of a permeable sub-population

All porin mutants (*E.coli* W3110 Δ*ompC*, Δ*ompA*, Δ*ompA*:Δ*ompC*, Δ*LamB*) and the wild type were grown in glucose limiting conditions, i.e. chemostat mode $0.1h^{-1}$ dilution rate, to observe how the permeable phenotype proportion evolves over time. Each cultivation was conducted in triplicate for 50 hours. Time zero corresponded to the drop in oxygen at the end of the batch phase leading to the switch to chemostat mode cultivation, it also marked the first sampling of the bioreactor for FC analysis.
Flow cytometry analysis of PI stained samples performed every 10 hours confirmed that porin deletion has an impact on the permeable sub-population abundance.

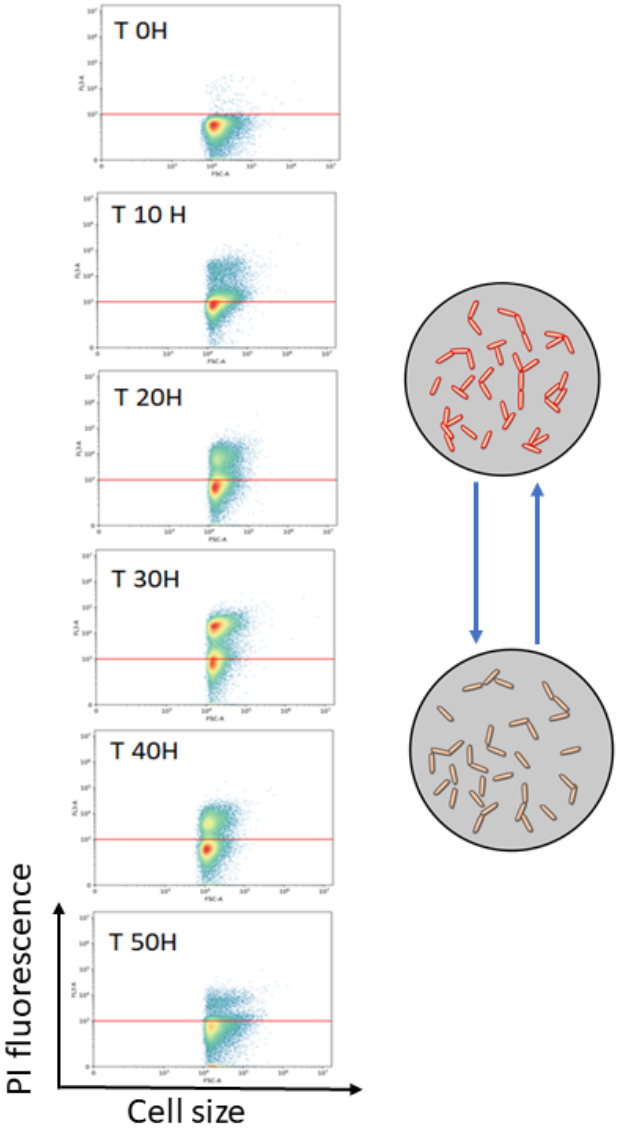

Figure 10: Flow cytometry analysis of *E.coi* Δ*ompA*: Δ*ompC* reveals bistability with a subpopulation partially stained by PI and another one that is unstained. The scales of both axis are logarithmic and Y axis (FL3-A channel) represents the fluorescence intensity while X axis (FSC-A channel) displays events sizes.

Firstly, it was observed that not all porin deletion resulted in identical increase in abundance of the permeable phenotype. While the wild type and Δ*ompA* did not experience permeability ratio above 10%, the double deletion (Δ*ompA*:Δ*ompC*) had a strong impact and clearly led to bistability (figure 10) with the permeable subpopulation representing half of total population after 30 hours.

Deletion of *ompC* and *LamB* resulted similarly with a permeability fraction topping respectively at 20 and 18 % (figure 11). It was also observed that with our FC resolution, distinction between the two subpopulations could not be made based on morphological characteristics (annex 9.2).

Secondly, the leaky fraction was found to vary over time (figure 11). Beginning from low values at the end of the batch phase, the abundance of the permeable phenotype peaked near 20% after ten hours for *E.coli* W3110 Δ*ompC* and Δ*LamB*. It should be pointed out that since samples were only taken every ten hours, it is likely that permeability did not peak after ten hours.

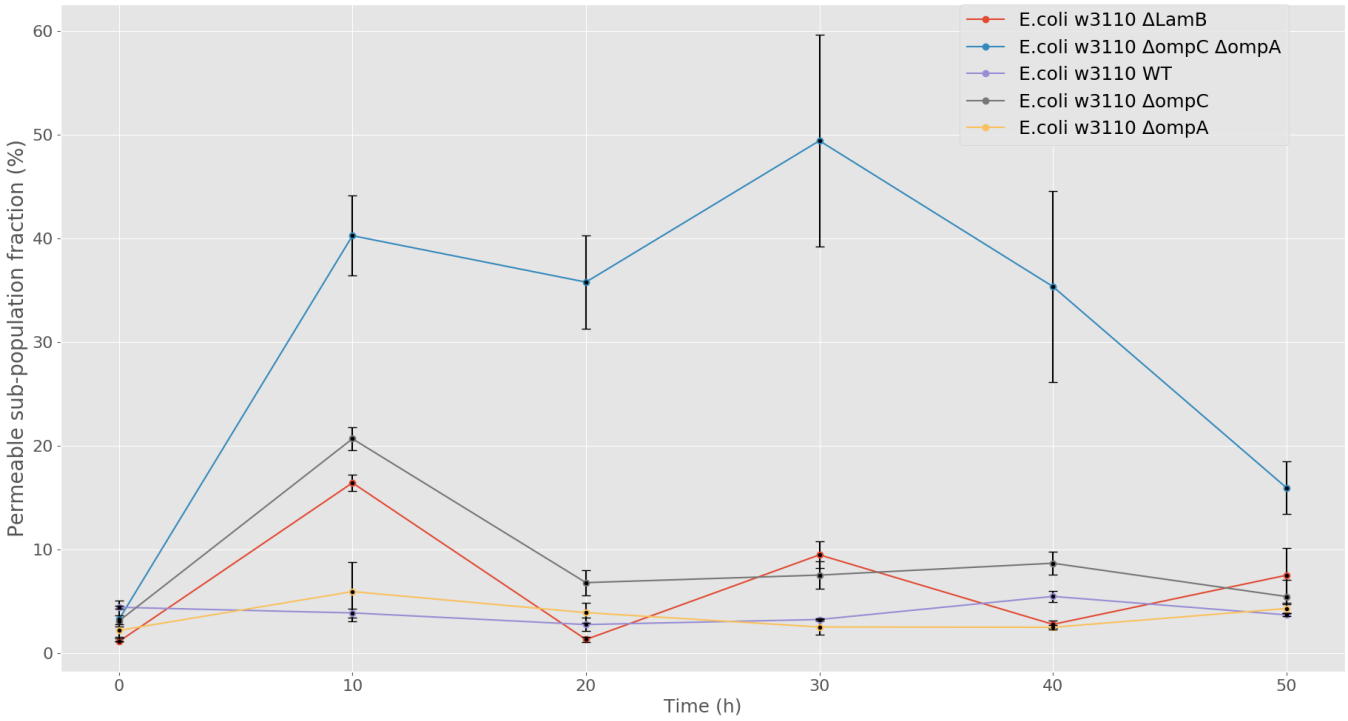

Figure 11: Monitoring of the outer membrane permeable phenotype proportion over time in glucose limiting conditions has revealed the dynamic nature of the two subpopulation partitioning

After 30 hours, the permeable phenotype even reached half of the overall population for the double mutant (Δ*ompA*:Δ*ompC*) before dropping steadily.

6.2 Permeable cell subpopulation exhibits a non monotonic dynamics and can be controlled by glucose pulsing

Even though glucose pulses (1 pulses/hour) performed by Thai Nguyen increased the fraction of the permeable phenotype of E.coli W3110 ΔompC, the dynamic remains identical to the one in non-pulsed chemostat.

The evolution of the permeable cell fraction (%) was monitored starting from the inoculation and during 60 hours.

Clearly the overnight preculture had an important ratio of permeable cells. Then over the glucose rich batch phase, this proportion declined before rising back up with the start of the glucose limitation (figure 12).

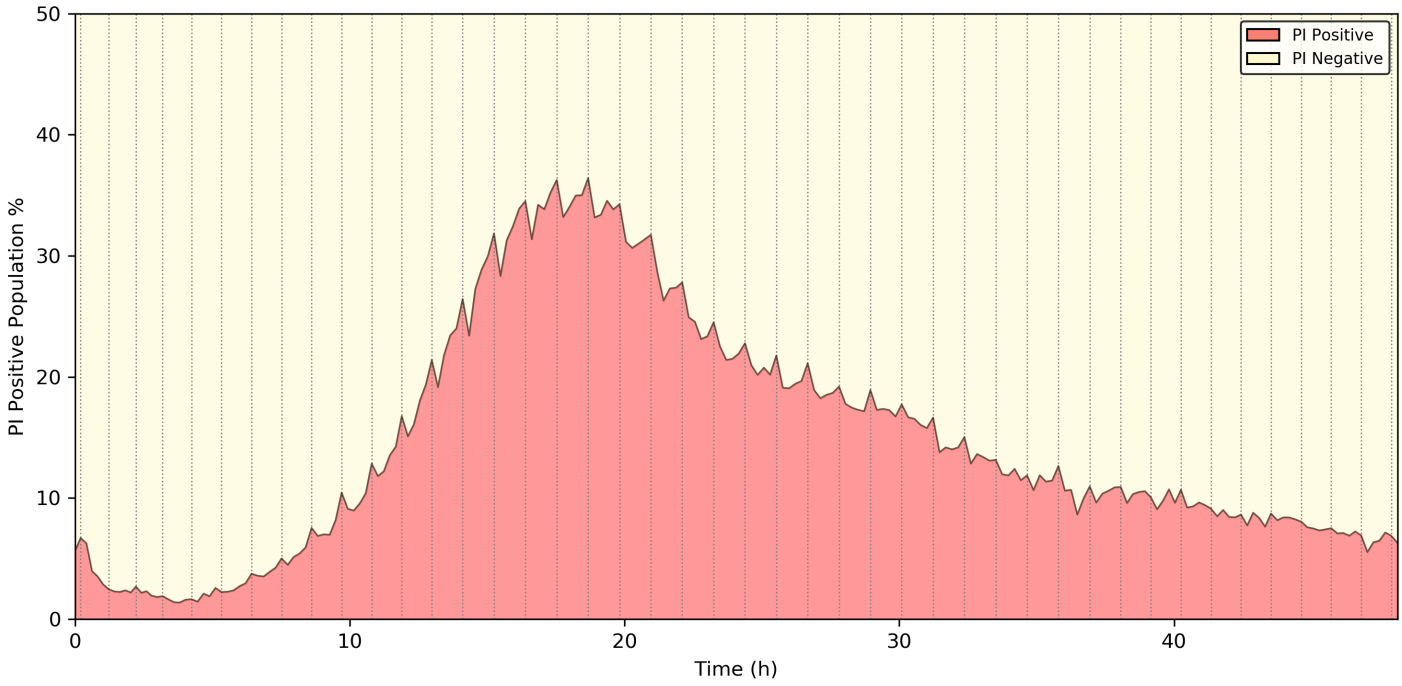

Figure 12: Impact of glucose pulsing on permeable subpopulation dynamics of *E.coli* W3110 Δ*ompC*

By comparison with the experiment conducted without pulses (section 8.1), the fraction of permeable cell went higher (25% instead of 20%) but again declined slowly. With each pulsing of glucose, the permeable phenotype abundance briefly decreased before jumping back up. Identically to the chemostat cultivation without glucose pulses, the population reached its maximal ratio of permeable cells around 10 hours after the start of the chemostat phase and then declined.

6.3 Evaluation of growth and morphological characteristics in microfluidics under glucose limiting and non limiting conditions

Having shown that permeability could be increased by removing porins and pulsing glucose to modulate the stress, microfluidic experiments were conducted to see if this increased permeability resulted in better growth under glucose limiting conditions. All of the mutants and the wild type were grown inside of a microfluidic cultivation device both with non limiting and limiting glucose concentrations. Time lapse microscopy revealed how the different stains growth and morphology was impacted. This first was measured by manually counting the number of cells every hours and during nine of them (annex 9.5). For each conditions and stains, four cultivation chambers were analysed (except for Δ*ompA* in non limiting conditions were n=3).

6.3.1 Impact of glucose deprivation on growth

By comparing the mean growth of each porin lacking mutants with the wild type, a t-student test (annex 9.3) revealed the fitness gain associated to porin removal in glucose limitation.

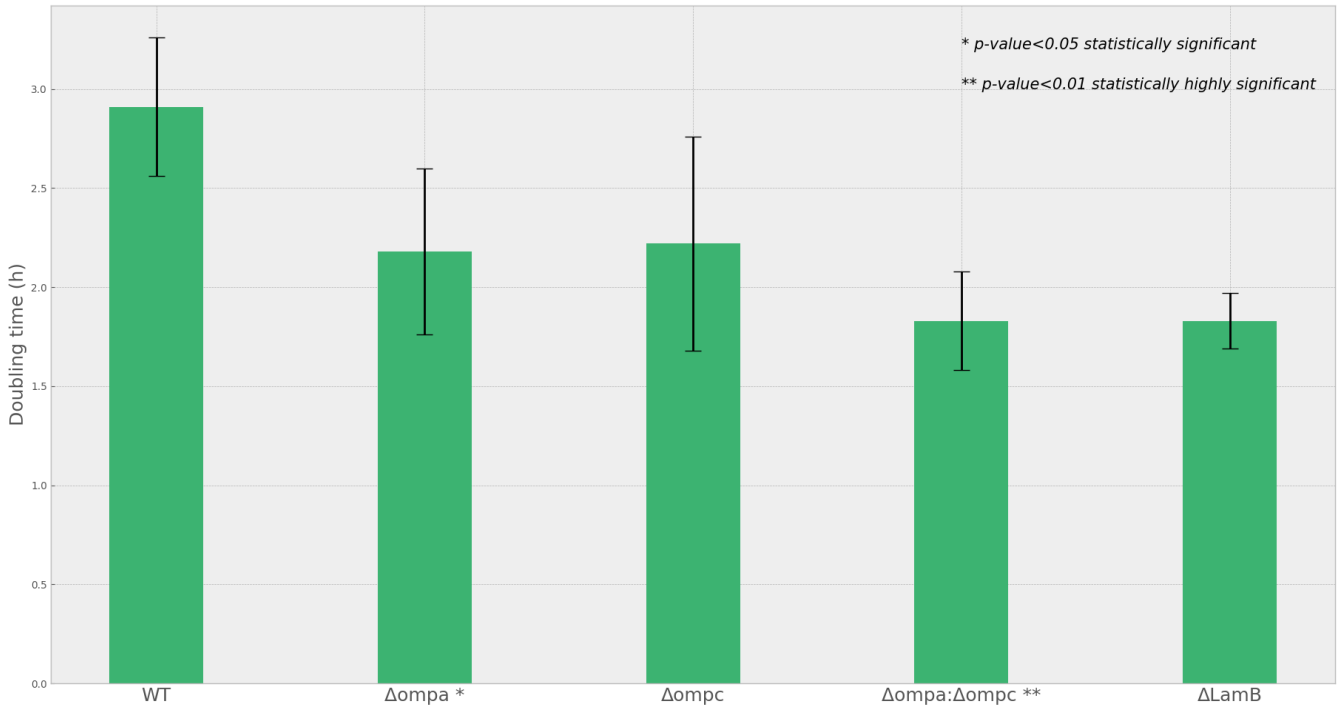

Figure 13: Influence of porin suppression on doubling time in glucose limiting conditions. Except ΔompC, all porin lacking mutants grew faster than the wild type in glucose limiting conditions

When glucose is limiting, with the exception of Δ*ompC*, all other porin deletion resulted in better growth with doubling time either significantly different, Δ*ompA*, or highly significantly different, Δ*ompA*:Δ*ompC* and Δ*LamB*, than the wild type (figure 13).

The same experiment and analysis were conducted in non limiting conditions to evaluate if this fitness gain did not come at a cost of inferior growth compared to the wild type when glucose was abundant. A *t test* and its derived *p-value* (annex 9.4) revealed that *E.coli* W3110 Δ*ompC* and Δ*LamB* did not exhibit any significant difference in growth with the wild type (figure 14). On the contrary, deletion of *ompA* negatively impacted growth since both Δ*ompA* and Δ*ompA*: ΔompC grew significantly(*p-value<0.05*) slower than the wild type.

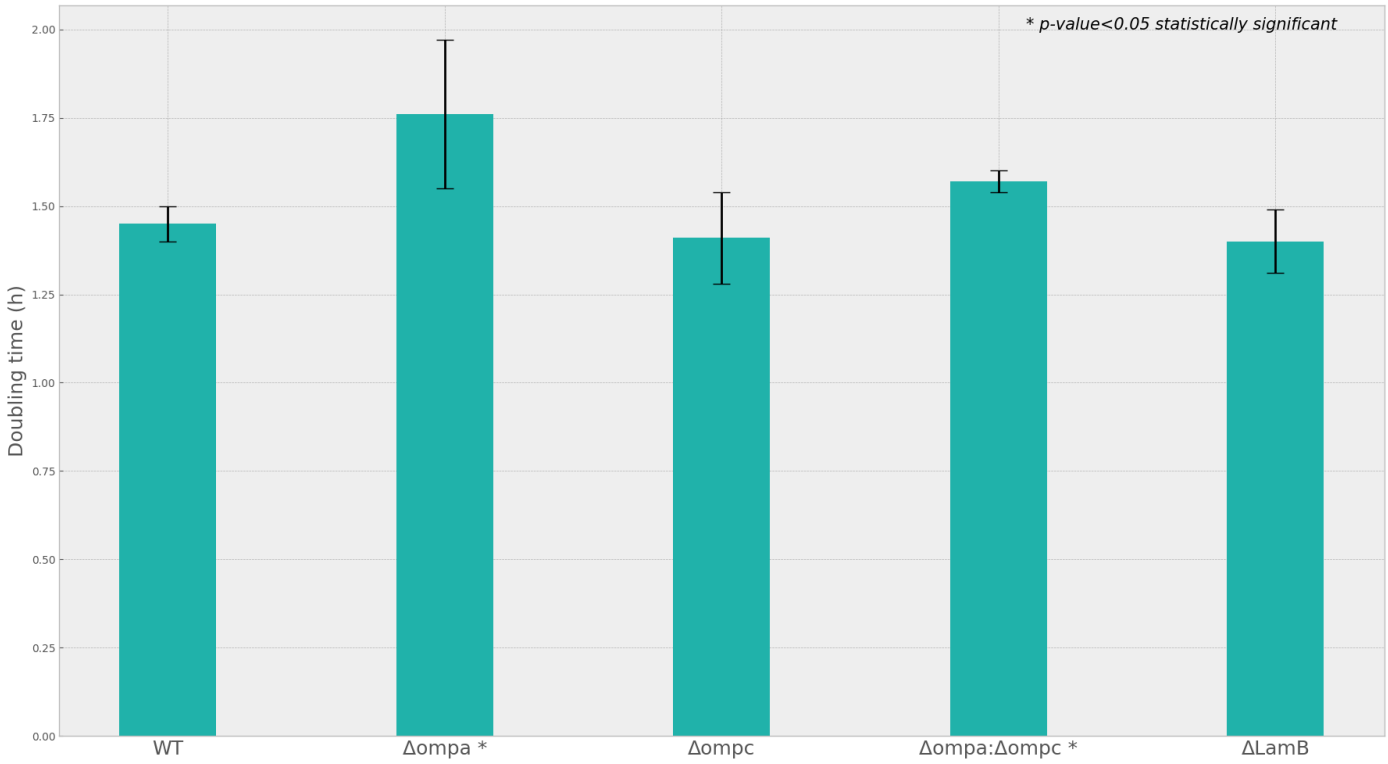

Figure 14: In glucose rich conditions, porin removal provides no advantages in growth compared to the wild type

These results demonstrated that porin removal does not automatically benefit the bacteria. While in limiting conditions, porin suppression almost always leads to increased fitness, these deletions had no beneficial consequences when glucose was abundant. On the contrary, some of these deletions detrimentally impacted growth in glucose rich conditions. Additionally to providing information on growth, microfluidics also revealed differences in cell size evolution and morphology that could help to get a broader picture on the impact of porin deletion and its associated trade-off.

6.3.2 cell size evolution and morphology

Cell size evolution during one cellular division cycle was evaluated. Bacterial size was shown to depend on stain, glucose conditions and cultivation duration.
Surprisingly, at first cells were growing as if no shortage of glucose was felt. Then, and for all mutants as well as for the wild type, growth declined and greater differences between the mutants appeared (figure 15). Taking into account this observation, cell size over one division cycle was evaluated both at the start (one hour after beginning cultivation) and near the end (six hours after beginning) of the cultivation.

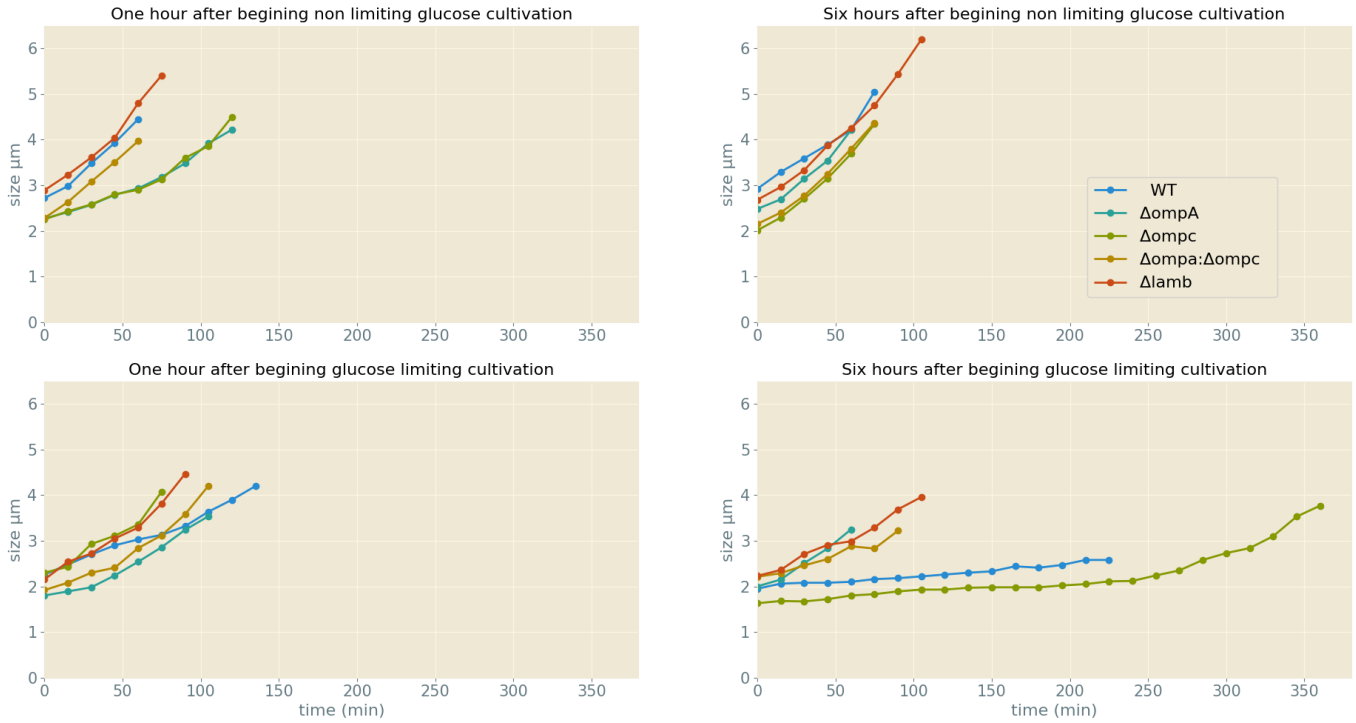

Figure 15: Overall representation of cell size evolution over one division cycle for each stains in glucose limiting and non limiting conditions

In light of these results it is clear that porin removal has an impact both on growth and on cell size evolution. Over one division cycle, all mutants were not born with the same size nor did they divided with identical length.

Firstly, cell size reached before division was negatively impacted by glucose shortage (figure 16). Although this was always true, the magnitude depended on the mutant. The wild type was particularly impacted by this nutrient stress that induced approximately a 50% drop in cell size before division compared to non limiting conditions.
Secondly, as the cultivation ages, the phenomena was amplified and cell division duration of *E.coli* wild type and $\Delta ompC$ dramatically increased.

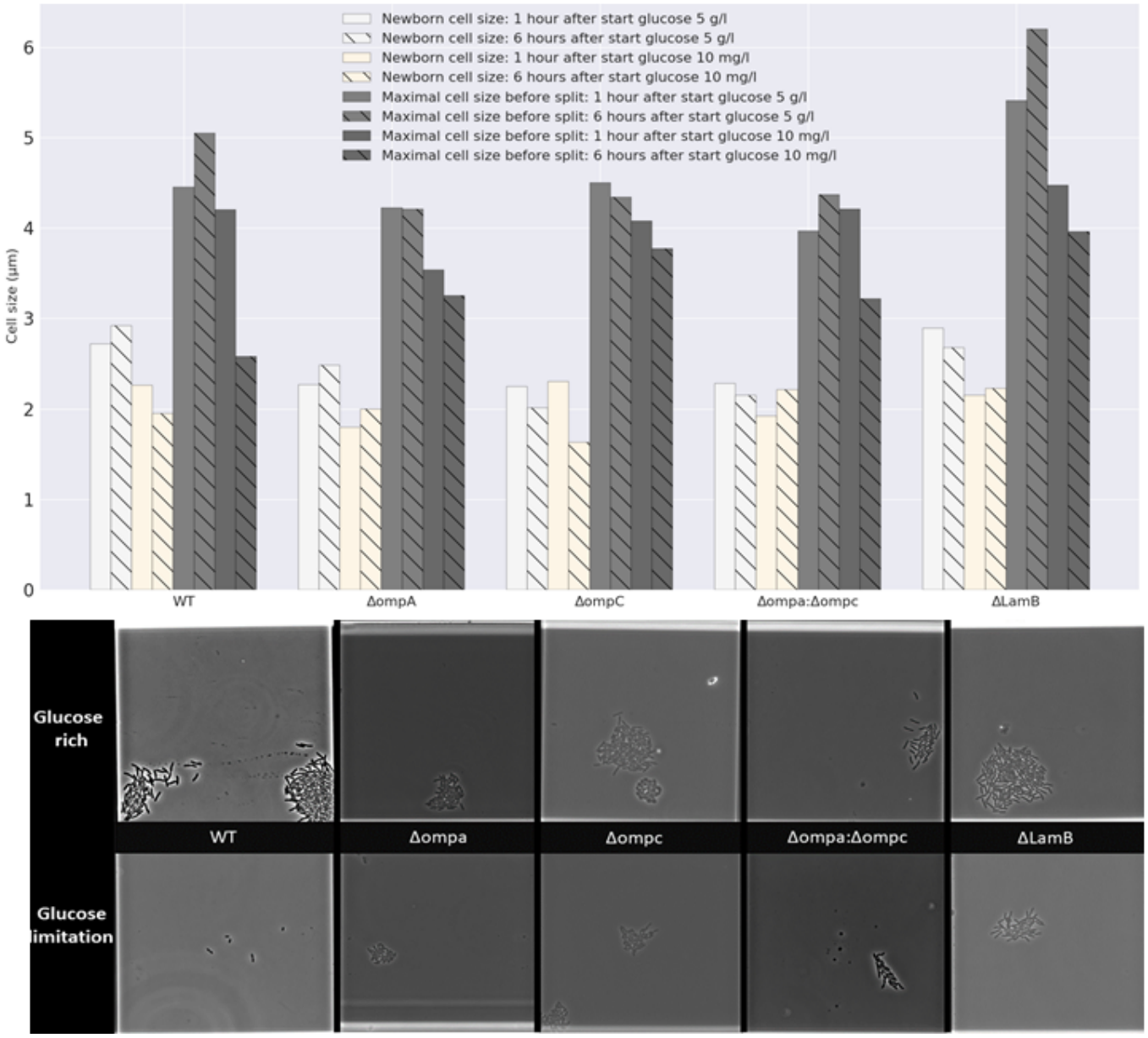

Figure 16: Influence of glucose conditions, cultivation duration and porin suppression on newborn and full-grown cell size. All images were taken after 6 hours of cultivation and show that in glucose limitation, bacteria a fewer and smaller.

Thirdly, as well as older bacteria and in a lesser extent, newborn cell size was also negatively impacted by glucose deprivation. The consequence of glucose limitation on newborn cell size was not as universal (not observed for Δ*ompC* and Δ*ompA*:Δ*ompC*).

Certain porin removal also greatly impacted the morphology and viability of the bacteria. Surprisingly, while both the deletion of *ompA* and *ompC* resulted in severe alteration, the double mutant did not exhibit any apparent alteration (figure 17).
In the case of Δ*ompC*, protoplasts have been observed both in limiting and non limiting conditions. The deletion of *ompA* appeared to result in a significant loss of fitness since lysed cells were frequently observed both in limiting and non limiting glucose conditions.

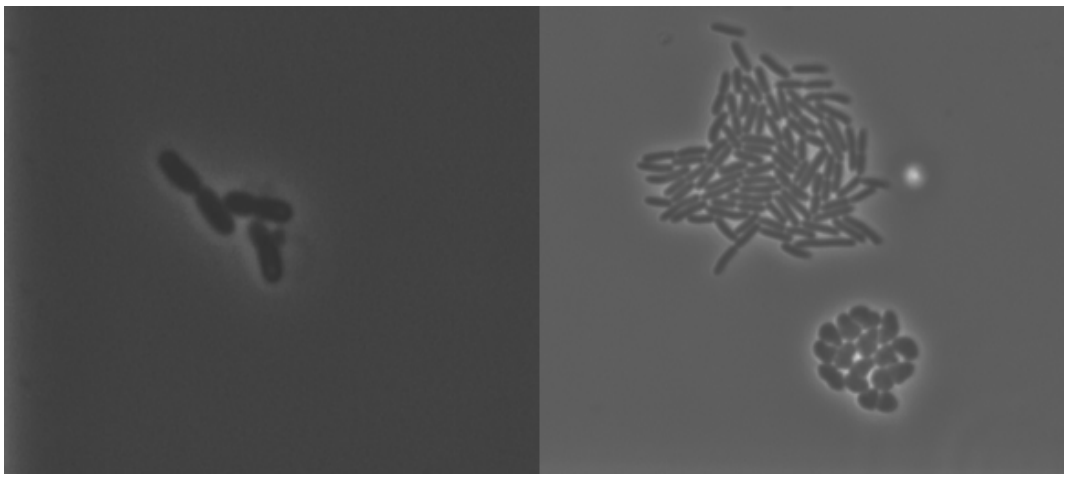

Figure 17: To the left Δ*ompA* lysed cells to the right Δ*ompC* protoplast

In a nutshell, glucose shortage often led to smaller newborn cells that always divided when reaching a smaller dimension than their unstressed equivalent. Moreover, the stress itself was not the only factor leading to a decrease in maximal cell size. Stress duration acted in the same direction and then, also negatively impacted maximal cell size.

Glucose deprivation impacted differently the strains. Were the wild type experienced important division cycle differences, bacteria size of Δ*ompA*:Δ*ompC* did not seems to be as affected. But not all mutants displayed an identical cell size in glucose rich conditions to begin with. The double mutant had an overall smaller size than the wild type, and quite notably, Δ*LamB* was surprisingly long and thus, even if maximal cell size experienced a drop due to glucose limitation, it still had a size similar to the one of the wild type in un-stressed conditions. Additionally, as the stress lasted, and compared with the other stains, the division time of Δ*ompC* and of the wild type sharply increased. This observation has led to the hypothesis that not all mutants had similar stock of energy at the end of the preculture.

6.3.3 Glycogen content of the wild type and porin lacking stains at the end of the preculture

Glycogen acts as an internal storage of energy for *E.coli* [Lanham et al., 2012]. It was hypothesised that the faster growing behaviour of some of the stains at the start of the glucose limiting cultivation came as a result of a higher glycogen accumulation during the preculture. This would explain why in limiting conditions Δ*ompC* and the wild type had such a fast growth at the start of the cultivation before considerably slowing down.

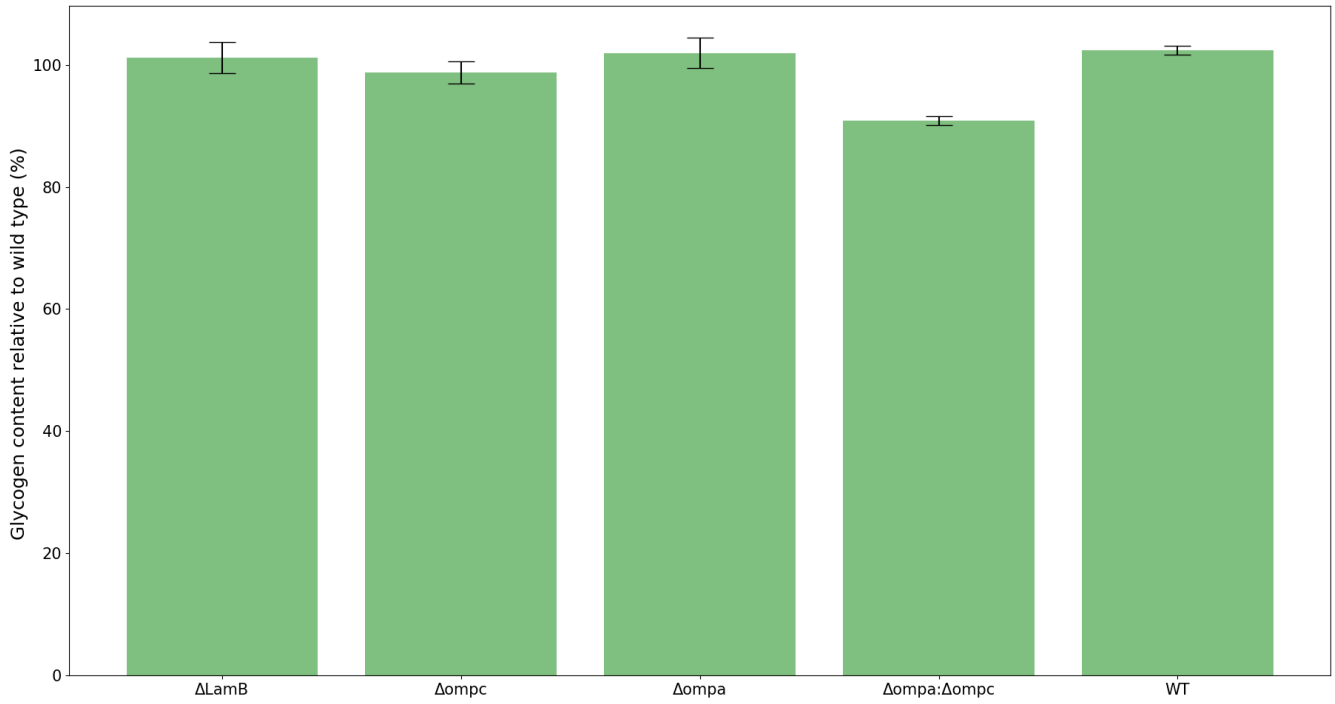

Figure 18: Relative glycogen content of each strain at the end of preculture is similar

Glycogen hydrolysis yield is unknown but all hydrolysis took place in parallel so by comparing the resulting glucose released by the reaction (annex 9.6), any major difference in glycogen accumulation should be visible. Intracellular glycogen content of each porin lacking mutant was expressed as a % of difference in glucose between the wild type and the analysed strain. The analysis revealed that glycogen accumulation was rather constant among strains with only minor differences (figure 18).

6.4 Evaluation of the potential role of eDNA in PI partial staining

Out of the scope of the study of the bet-hedging associated to the bistability observed in glucose limiting conditions, propidium iodide staining was shortly investigated. As previously mentioned, propidium iodide staining of *E.coli* living cells is not understood. One tangible hypothesis was that PI binds to more permeable cells because of the presence of extracellular DNA (eDNA). If so, a Dnase treatment should result in a significant decrease in proportion of the partially stained sub-population.

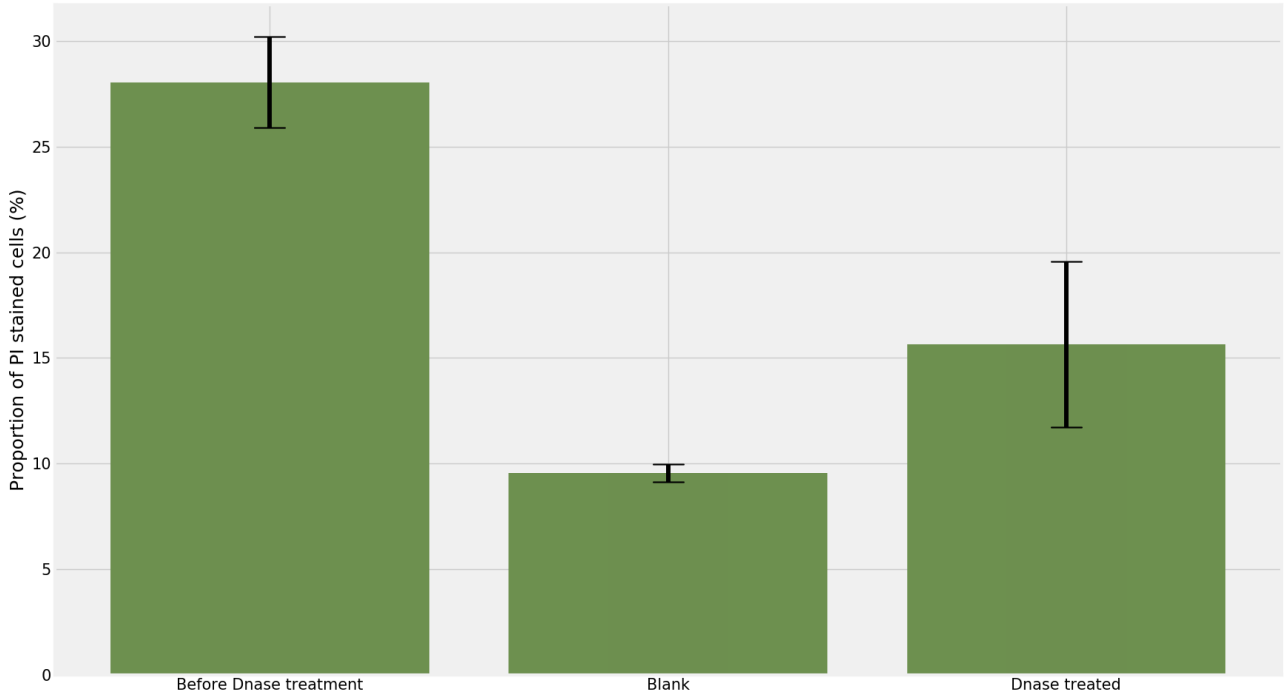

Figure 19: Influence of Dnase treatment on the proportion of PI stained sub-population
The duration and or temperature of the treatment itself had an impact on the staining and Dnase showed no influence

Flask cultivation of *E.coli* W3110Δ*ompA*:Δ*ompC* were realised in triplicate. As presented in figure 19, twenty hours after cultivation start, the population was composed of (28%) partially stained cells. Samples from these flask cultivation were Dnase treated for two hours at $37°$c before being stained with PI. Blank analysis consisted in cells being incubated with the Dnase buffer and without Dnase were also realised. First, it was observed that propidium iodide staining was not stable. Samples had to be analysed fresh or the proportion of partially stained cells decreased. This phenomena was clearly showed here, during the two hours of the Dnase treatment, even the blank suffered an important drop in its PI stained proportion. Secondly, Dnase treatment did not led to any noticeable drop in the partially stained proportion. Thus, it is unlikely that eDNA is the causes of the partially stained phenotype.

7 Discussion

7.1 Porin deletion exacerbates outer membrane permeability

Studies about phenotypic diversification of microbial populations are attracting a lot of attention because of the possible new functionalities exhibiting diversified populations. Division of labour or bet hedging increasing the global fitness of the whole population with important consequences like persister cells following antibiotic treatment or non-producing subpopulations in bioprocessing. Effective control of phenotypic diversification of microbial population requires the use of advanced technique and/or modelling for the effective spatio-temporal characterisation of the population. In suspended culture, the spatial contribution is given by cell to cell differences, requiring the definition of an appropriate single cell proxy. In this work, we have used PI as a proxy for membrane permeabilization. We have confirmed the occurrence of a red but not dead (RBND) phenotype and excluded possible side-effects/artifacts in the detection of this phenotype, i.e. binding of PI to eDNA and effect of storage compounds (glycogen) on the extended growth phenotype under glucose limitation.

In line with previous observations made on the knockout porins mutants from the Keio library, porin suppression has been confirmed to increase the occurrence of a the partially stained phenotype (unpublished results). Although the mechanisms behind this increase in OM permeability are unknown, changes in outer membrane porins composition is hypothesised since porin removal has been shown to up and down-regulate other porins expression [Yang et al., 2011]. Also, because some of the porins such as OMPA are highly abundant, i.e. at around 100 000 copies per cell [Molloy et al., 2000], their removal leaves room to proteome re-allocation. Thus, it is speculated that porin removal increases the ratio of PI positive cells by inducing the appearance of larger porins in the outer membrane. While only a proteomic analysis would answer this hypothesis, a thorough understanding of PI partial staining is primordial to fully understand the nature of this phenotype.

7.2 Increase in membrane permeability leads to improved growth fitness in glucose limiting conditions

Porin mutants were utilised because such deletion have been shown to increase the permeable (PI positive) subpopulation and was thought to ease the observation of bet-hedging. This increased fraction of permeable cells systematically results in better performances when glucose in scarce. And although increased fitness confirms that this bistability is a bet-hedging mechanism, results have been influenced by the mutations themselves (figure 20).

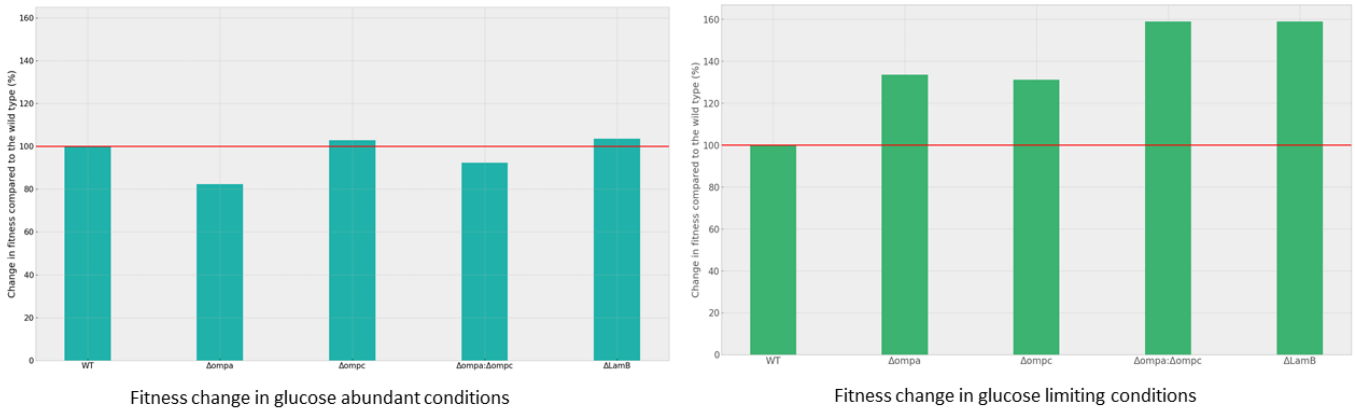

Figure 20: While porin suppression decreased fitness in glucose rich conditions, most of the mutations increased fitness compared to the wild type under glucose limitation. Fitness is expressed as the specific growth rate of each strain divided by the specific growth rate of the wild type

This was more particularly observed for the mutants exhibiting deletion at the level of the OMPA porin, a very important OM structural protein. As a result, deletions of *ompA* and *ompA*:*ompC* have negatively impacted bacteria fitness in glucose rich conditions thus adding complexity to the phenomenon.

7.3 Dynamics of permeabilized subpopulation is non-monotonic and involves two distinct adaptive strategies

By monitoring the abundance of the permeable phenotype over time we have observed that the strategy adopted by a population to face nutrient stress is dynamic and occurs at different time scales (figure 21). The dynamics can be split into three characteristic times partially overlapping. Firstly, it was observed that pulses of glucose induce a fast and non lasting decrease of the permeable (PI stained) subpopulation of cells. This fast response to glucose pulses is mainly observed while the population is globally increasing in permeability, i.e. between 5 and 19 hours of cultivation.
Bet-hedging is observed during the increase in permeability of the population and represents a first strategy to face nutrient stress. Then the permeable fraction steadily decreases and the population adopts another strategy. Although microfluidics experiments did not allow for the observation of permeability according to PI staining, as the glucose stress lasted a decrease in cell size was observed. Thus, we propose two hypotheses to explain the decrease in permeability. First, because smaller bacteria have been shown to appear when limitation continues, we hypothesise that these smaller cells would not be stained and compete with the PI positive phenotype. The small cell phenotype would then succeed to the partially stained one and gradually take over as a slow adaptive strategy. In this scenario, the partially stained phenotype would be a first answer to glucose stress before a second strategy that requires deeper morphological changes takes over by competing.

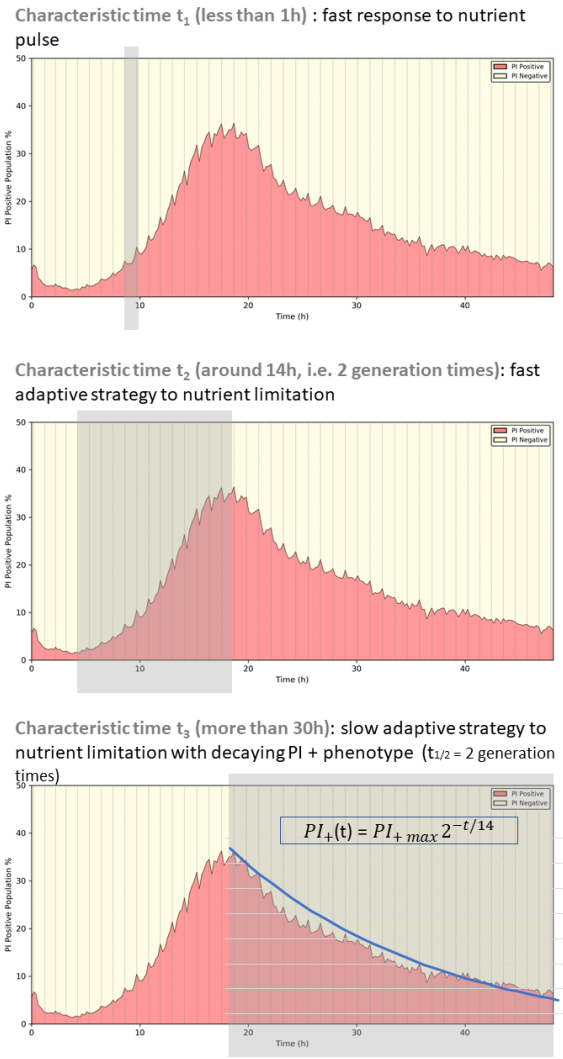

Figure 21: *E.coli* adaptation to nutrient stress is non monotonic and involves a succession of adaptive strategies. One hypothesis is that the decrease in PI positive ratio would follow an exponential decay with a half life of 2 generations (around 14 h).

The second hypothesis to explain the dynamic observed relies on the renewal of the outer membrane instead of competition. It is know that between the addition of a OMP in the membrane and the appearance of new bacteria that contain none of these OMPs, two generations are needed (figure 22) [Rassam et al., 2015]. The ratio of partially stained bacteria began to decrease after a duration that closely corresponds to two generation times, i.e. one generation time equal $\ln(2)/\mu$ (dilution rate=μ =0.1 $h^{-}1$) so two generation times roughly correspond to 14 hours under the operating conditions considered.

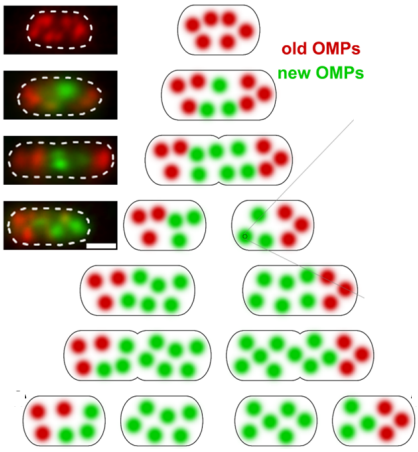

Figure 22: OMPs are pushed away to the poles of the cell for the renewal of OM composition. According to this mechanism, two generations are needed for the appearance of bacteria with a totally renewed OMP composition (adapted from [Rassam et al., 2015])

Thus, the appearance of this partially stained phenotype would again be a first response of the population to glucose limitation. The consequences of this bistability would be perceived during two generations because it corresponds to the time needed for new bacteria with a fresh OM composition to emerge. Microfluidics experiments have revealed that bacteria cell size is negatively impacted by glucose shortage duration. It is then hypothesised that this second strategy relies on a smaller bacteria size instead of higher permeability. But two generation are not enough to dilute all the OMs section that led to PI staining. The decrease in PI positive bacteria would follow an exponential decay with a half life of 2 generations (around 14 h). Because the duration of one generation depends on the dilution rate, using a model, it should be possible to predict when and with which rate the PI positive faction would decrease as a function of dilution rate. The theory of a two phenotypes succession could be verified by fitting the model to real data. Alternatively, the use of a FC with higher cell size resolution would enable to observe the decrease in cell size.

Part of the change in strategy could also be explain by the induction of Adenosine 3',5'-monophosphate (cAMP) synthesis. Indeed, cAMP regulates the transcription of many genes, including porins. cAMP acts as a glucose starvation stress indicator. When the PTS sugar substrate is scarce, then adenylate cyclase is strongly activated and generates cAMP. But the production of cAMP required ATP and then, if glucose limitation lasts, the production of cAMP declines[Lin et al., 2004]. The evolution of this permeable sub-population could follow the one of cAMP, meaning a high permeability at the end of the batch phase followed by a declined associated to the one of cAMP. Such theory could be tested by monitoring the intracellular content of cAMP. Direct addition of cAMP in medium when the permeable fraction declines could also help to challenge this hypothesis.

8 Conclusion

As an important conclusion, we have observed that chemostat cultivation mode fails at maintaining a truly stable population. A succession of two adaptive strategies has been observed and revealed that upon glucose limitation, *E.coli* population responses in non monotonic and involves several, phenotypically different, subpopulations. Our work as been focused on the occurrence of two subpopulations, i.e. a first one exhibiting a low OM permeability and a second one with high OM permeability. Although porin suppression has added complexity to the phenomenon, in glucose limitation, the permeable subpopulation seems to benefit from the increase outer membrane permeability, following a bet-hedging strategy.

However, during prolonged continuous cultivation this bet-hedging strategy is progressively replaced by other adaptive strategy involving drastic changes in cell size. Indeed, there are strong indications that a smaller and more adapted phenotype takes over and that the succession of these strategies is related to cell memory and more specifically, outer membrane turnover. However, the unknown mechanism by which PI binds to living bacteria hinders the characterisation of the partially stained phenotype. Although OM porin composition seems to influence and then explain this partial PI staining, other components of the outer membrane could be at the basis of the staining. All in one, this work point out the fact that several adaptive strategies involving key subpopulations of cells can take place simultaneously in continuous cultivation. This work pave the way for further understanding about population stability and phenotypic diversification under nutrient limitation. These knowledge will be exploited for controlling *E.coli* population based on the segregostat strategy introduced during the bibliographic section of this document.

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9 Annex

9.1 HPLC method description

- Eluant: H_2SO_4 5 mM 100% Isocratic
- Flow: 0.5 ml l^{-1} for 30 minutes
- Column and injection: 10 μl in a Aminex HPX-87H column at $40°$c

9.2 Permeable phenotype cannot be distinguished based on morphological characteristics (cell size) with low FSC resolution

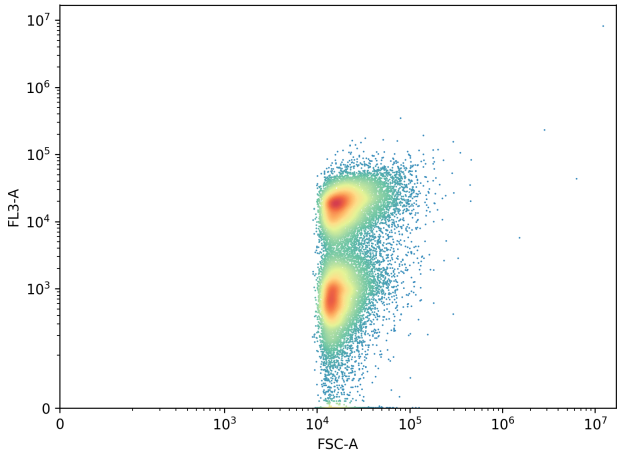

Figure 23: Δ*ompA*:Δ*ompC* t 30h PI cell staining as a function of cell size. Two subpopulations with different straining are present

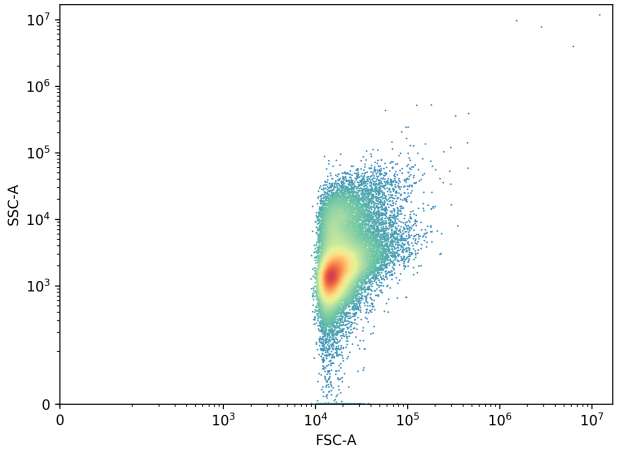

Figure 24: Δ*ompa*:Δ*ompc* t 30h cell internal complexity as a function of cell size. Two subpopulations cannot be distinguished based upon morphological characteristics

9.3 Statistical analysis of the impact of porin deletion on growth in glucose limiting conditions

Impact of porin suppression on growth in limiting glucose conditions			
E.coli W3110 WT	T-student	DF	p-value
E.coli Δ*ompC*	-2.145	6	0.0757
E.coli Δ*ompA*	-2.670	6	0.0370 *
E.coli Δ*ΔompA-ΔompC*	-5.022	6	0.0024 **
E.coli Δ*LamB*	-5.730	6	0.0012 **

9.4 Statistical analysis of porin removal impact on growth in glucose non limiting conditions

Impact of porin suppression on growth in glucose non limiting conditions			
E.coli W3110 WT	T-student	DF	p-value
E.coli Δ*ompC*	-0.574	6	0.5866
E.coli Δ*ompA*	2.872	6	0.0283 *
E.coli Δ*ΔompA-ΔompC*	3.643	5	0.0149 *
E.coli Δ*LamB*	-0.971	6	0.3689

9.5 Raw data microfluidic experiment: cell number over time

	WT 5g/l			WT 10 mg/l	
Time (minutes)	Mean number of cells	σ		Mean number of cells	σ
0	1	0		1	0
60	2	0		1,5	0,58
120	3,67	1,26		1,75	0,5
180	6,33	1,71		3	1,15
240	9,33	2,65		3,75	1,26
300	16,33	5		5,75	2,06
360	21,67	4,65		6,25	1,71
420	34	7,94		7,75	1,71
480	54	8,18		8,5	2,65
540	76,67	12,58		9	2,45
	Δompa-Δompc 5g/l			Δompa-Δompc 10 mg/l	
Time (minutes)	Mean number of cells	σ		Mean number of cells	σ
0	1	0		1	0
60	1,67	0,58		1,25	0,5
120	2	0		1,75	0,5
180	3,33	0,58		2,75	0,96
240	5,33	1,15		4,75	2,22
300	8,67	2,08		7,25	2,87
360	14	5,2		10	4,97
420	22	5,57		16	8,04
480	35,67	4,16		26,5	11,12
540	53,67	4,04		34,25	14,43
	Δompa 5g/l			Δompa 10 mg/l	
Time (minutes)	Mean number of cells	σ		Mean number of cells	σ
0	1	0		1	0
60	1,25	0,5		1,5	0,58
120	1,75	0,96		2	0
180	3	1,15		2,75	0,96
240	4	2,31		4	0,82
300	6	2,31		5,75	2,06
360	10,75	3,4		6,75	2,22
420	16,5	8,89		10	3,65
480	25,75	10,53		13,75	6,95
540	38,75	19,36		21,5	12,5

	Δompc 5g/l			Δompc 10 mg/l	
Time (minutes)	Mean number of cells	σ		Mean number of cells	σ
0	1	0		1	0
60	1,5	0,58		1,25	0,5
120	2,5	1		2	0
180	4	0,82		3,75	0,5
240	7,25	2,22		6,5	1,29
300	14	5,42		8,5	2,65
360	20,5	6,56		11,5	3,11
420	38,75	12,69		16,5	7,05
480	65,5	24,28		20,25	11,87
540	91	29,54		24,5	21,39
	ΔlamB 5g/l			ΔlamB 10 mg/l	
Time (minutes)	Mean number of cells	σ		Mean number of cells	σ
0	1	0		1	0
60	1,5	0,58		1,5	0,58
120	2,5	1		2	0
180	5	2		3,25	0,96
240	9,5	4,36		4,25	0,5
300	17,5	7,05		7,5	1
360	28,75	12,42		12	2,31
420	44,25	20,29		17	3,37
480	59	20,35		24,25	4,27
540	89,75	30,89		31,25	6,85

9.6 HPLC analysis of glucose content derived from intracellular glycogen hydrolysis

	Retention time (min)	Area
E.coli WT 1	8.271	3136806.6818
E.coli WT 2	8.802	3164048.1822
E.coli WT 3	8.619	3087193.9149
E.coli Δompa 1	8.323	3265642.3619
E.coli Δompa 2	8.532	3045953.9447
E.coli Δompa 3	8.515	3033429.7766
E.coli Δompc 1	8.270	2907877.4042
E.coli Δompc 2	8.439	3055577.1892
E.coli Δompc 3	8.492	3085926.8339
E.coli Δompa-Δompc 1	8.240	2732140.1134
E.coli Δompa-Δompc 2	8.499	2808335.6677
E.coli Δompa-Δompc 3	8.467	2782577.4634
E.coli ΔlamB 1	8.528	3244256.2109
E.coli ΔlamB 2	8.433	3044496.9171
E.coli ΔlamB 3	8.334	2983409.8031