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INSIGHTS ON CONTACT-DEPENDENT COMMUNICATION IN BACTERIA

MESTRADO EM BIOLOGIA MOLECULAR E GENÉTICA

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Abstract

Competition is a ubiquitous process in evolution. In the bacterial world many examples exist of mechanisms used by bacteria to outcompete other bacteria. Some of these mechanisms are triggered by complex intercellular communication. Quorum sensing signaling is the most well-known example of such communication. However, while quorum sensing is based on production, dissemination and detection of diffusible molecules, other communication mechanisms rely on direct cellular contact such as the contact-dependent growth inhibition mechanism in *Escherichia coli*. Here we characterize a new high density-dependent growth advantage signaling: when sensing other cells at high densities, bacteria increase their growth rate. Moreover we show that this new communication mechanism also occurs between different species of *Enterobacteriaceae* such as *Escherichia coli and Salmonella enterica* but seems to be lacking in the firmicute *Staphylococcus aureus*. After testing the supernatant of high-density grown cultures for any diffusible signal molecules that could be responsible for such communication, we concluded that the phenomenon of high density-dependent growth advantage only occurred when the focal cells were in contact with the high density competitor cells. For depending on cellular contact we call this new bacterial growth promotion mechanism, the contact-dependent growth advantage. After analyzing the growth of 56 *E. coli* mutants for genes encoding outer membrane proteins we found a mutant for the bacterial cellulose synthesis gene, *bcsC* that did not display such advantage. Although the successful complementation of *bcsC,* there was no contact-dependent growth advantage phenotype rescue. However, *pqqL,* a gene playing a putative role in iron uptake, is a good candidate to understand this mechanism, indicating a putative link between iron uptake and the contact-dependent communication system here described. In fact, further investigation on this topic would be important in the clinical context since the molecular regulation of such mechanism could be the key to modulate some bacterial infection processes.

Keywords: Bacterial competition; Contact-dependent communication; Signaling; Growth advantage; Iron uptake.

Resumo

Durante muito tempo as bactérias foram estudadas sob a premissa de responder aos estímulos externos de forma individual. Aspectos como virulência e resistência a antibióticos, tão importantes no contexto clínico, foram muitos anos investigados sem ter em conta qualquer tipo de comunicação mais complexa que pudesse existir no mundo microbiano. Contudo, descobriu-se mais tarde que as bactérias comunicam através de uma "linguagem própria", à qual foi dada o nome de *quorum sensing*. Esta comunicação, baseada na produção e difusão de moléculas sinalizadoras, permite que as células bacterianas infiram a densidade celular no meio que as rodeia. Com essa informação são capazes de alterar a sua expressão génica dando origem a comportamentos ao nível de grupo, só assim sendo possível dar uma resposta adequada a certos estímulos ambientais. Existem, contudo, outros mecanismos responsáveis pela comunicação entre bactérias que dependem do contacto directo entre células. Um dos casos mais conhecidos é o da espécie bacteriana Gram negativa *Myxococcus xanthus* que, através do contacto directo célula a célula, dá início a um processo complexo de desenvolvimento que leva à agregação celular e subsequente estrutura multicelular. Deste modo *M. xanthus* é capaz de dar resposta a estímulos como escassez de nutrientes e simultaneamente eliminar competição directa, sendo capaz de predar outras bactérias. Recentemente um novo tipo de mecanismo envolvendo contacto celular foi descrito em *Bacillus subtilis.* Este novo mecanismo é baseado na existência de nanotubos que permitem a troca intercelular de metabolitos, ácidos nucleicos e outras substâncias importantes para a célula. Por sua vez em 2005 Aoki *et al.* descobriram um novo tipo de competição entre bactérias mediado pelo contacto: através da interacção entre proteínas da membrana externa, *E. coli* é capaz de produzir e transferir uma toxina para o citoplasma das células competidoras. Essas toxinas vão então interromper a produção de ATP, o que leva à inibição do crescimento das células alvo. Com a existência destes exemplos de comunicação e interacção celular por contacto directo o paradigma da comunicação bacteriana começa a ser visto com mais interesse científico.

Num estudo anterior, Nunes (2012) verificou a existência de um mecanismo desconhecido, dependente de alta densidade celular, que confere a *E. coli* uma maior vantagem adaptativa *(fitness)* em fases iniciais do crescimento. Nesse estudo foi também sugerido que esse mecanismo dependia do contacto célula a célula sem, no entanto, identificar os genes envolvidos neste tipo de comunicação bacteriana. Como tal, o objectivo desta tese passa por melhor caracterizar este novo tipo de comunicação intercelular, confirmando a necessidade de contacto celular e explorando o mecanismo molecular envolvido.

Usando *E. coli* como organismo modelo, os nossos resultados mostram que uma população constituída por um número muito baixo de bactérias cresce mais depressa quando em competição com outra população em alta densidade. Adicionalmente também mostramos que este tipo de comunicação não só ocorre entre células de *E. coli,* mas também entre *S. enterica* e *E. coli.* Contudo o mesmo não acontece entre *S. aureus* e *E. coli* nem entre *S. aureus e S. aureus*.

Posteriormente, confirmámos que a vantagem no crescimento não só depende de alta densidade celular, como também da dependência do contacto celular uma vez que não há influência do autoindutor responsável pelo *quorum sensing* entre diferentes espécies de Gram-negativas, isto é, o AI-2. Excluímos também a hipótese de existirem outros metabolitos ou moléculas sinalizadoras excretados pela população em alta densidade que pudessem ter efeito na vantagem no crescimento.

Para estudar o mecanismo de sinalização subjacente à necessidade do contacto celular recorremos a uma lista de 56 mutantes de genes codificantes de proteínas da membrana externa de *E.coli* e testámos para cada mutante o comportamento relativamente à vantagem no crescimento que aqui reportamos. Para tal, avaliámos o crescimento, em simultâneo, de cada mutante com uma estirpe selvagem, em baixa e alta densidade com o objectivo de identificar os mutantes que apresentavam um menor *fitness* relativa em alta densidade. De entre os 56 mutantes testados, os mutantes para os genes *bcsC, pqqL* e *yncD* são os que apresentam essa característica. De facto, os mutantes Δ*bcsC e* Δ*pqqL* não apresentam qualquer vantagem significativa no crescimento quando em contacto com uma população em alta densidade celular. O mesmo não se passou com o mutante Δ*yncD* que se comportou da mesma maneira que a estirpe selvagem.

O gene *bcsC* codifica uma proteína da membrana externa envolvida na exportação de celulose, um polissacárido envolvido na formação de biofilmes. Como tal, estando a produção de celulose associada a um maior número de contactos celulares, o produto do gene *bcsC* poderia ser responsável pela sinalização envolvida no fenómeno da vantagem no crescimento dependente do contacto. No entanto, de acordo com a literatura, a estirpe laboratorial *E.coli* K12 não sintetiza celulose o que nos levou a colocar a hipótese de que a proteína BcsC pudesse ter uma outra função. Contudo após termos procedido à complementação do gene *bcsC* na estirpe mutante, o fenótipo de vantagem no crescimento dependente do contacto não foi resgatado. Adicionalmente, a delecção do mesmo gene num *background* diferente da *E.coli* K12 também não teve influência nesse fenótipo.

Por sua vez o gene *pqqL* codifica uma peptidase com função desconhecida. Alguns estudos mostram no entanto que este gene poderá fazer parte de um novo mecanismo envolvido na

captação de ferro sendo um dos genes cuja expressão é aumentada em condições limitantes deste metal, necessário para o crescimento bacteriano. De facto, a captação de ferro é um dos mecanismos mais activados durante a fase *lag* bacteriana o que pode explicar o fenómeno de vantagem no crescimento aqui descrito: quando em baixa densidade as bactérias teriam uma expressão basal de genes envolvidos na captação de ferro tendo por isso uma determinada fase *lag.* No entanto, em alta densidade, devido ao contacto entre células, um mecanismo de sinalização seria accionado de modo a aumentar a expressão génica de genes envolvidos na regulação do ferro, o que poderia dar origem a uma fase *lag* mais curta. Deste modo, uma população em baixa densidade atingiria mais cedo o crescimento exponencial numa situação onde a competição por nutrientes é alta o que lhe conferiria uma grande vantagem evolutiva. Até à data de conclusão desta dissertação, não foi possível testar a hipótese supramencionada.

Em estudos posteriores pretendemos comprovar o papel do gene *pqqL* neste novo tipo de comunicação em *E. coli* dependente do contacto através do restauro do gene no respectivo mutante e subsequente resgate do fenótipo. Ensaios com outros genes envolvidos na captação do ferro e a análise da variação da expressão génica desses genes em função de diferentes densidades celulares, seriam também importantes para conhecer melhor este novo mecanismo de comunicação. Tal conhecimento poderá vir a tornar-se relevante no contexto clínico trazendo novas alternativas à modulação de diversos processos de infecção bacteriana por parte de estirpes patogénicas.

Palavras-chave: Competição; Comunicação; Vantagem adaptativa; Crescimento bacteriano.

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INTRODUCTION

"Struggle for existence"! These are the words chosen by Charles Darwin to explain the Theory of Natural Selection (Darwin, 1859). According to this theory, variation among individuals of the same species arises by chance and then some are preserved because they help the survival and reproduction of their carriers. Even if a population is very well adapted, a new variant individual may be even more adapted. This means that this individual, the carrier of a new adaptive mutation, has higher probability of reproducing (this may occur for many reasons: this variant may be more resistant to a disease, or stronger hence more able to run from predators and to run after preys, better sense of smell, among many other causes). If this new variant is more successful to reproduce than the rest of the population, it is leaving more descendants then conspecifics. Assuming that the population is close to its carrying capacity, the success of some individuals implies the failure of others: struggle for existence.

There are several mechanisms to deal with the other members of the population. Bacteria have found many ways to compete with other cells. Some bacteria establish positive relationships with other individuals, such as in bacterial biofilms. Others, however, are more spiteful, spending some of their resources to produce bacteriocines or antibiotics (Cascales et al., 2007). Some bacteria may even use bacteriophages as a weapon to kill other bacteria, as recently shown in our laboratory (Gama et al., 2013).

Contrary to the fundamentals of evolutionary theory, in the beginning microbiologists studied bacteria mostly as individual, non-interacting, entities. Thus, the great slice of investigation in bacteria focused on individual aspects rather than paying attention to intercellular communication and multicellular behavior. Today it is known that bacteria communicate and exchange information in many different ways (Brameyer et al., 2015; Greenberg, 2003; Waters and Bassler, 2005). Some of them are intriguingly complex and prove that there still a long way to run to better understand the language of bacteria. This knowledge might be proven very helpful in ecological and clinical context. Through this communication, bacteria are able to alter their gene expression and coordinate complex behaviors at the multicellular level. These behaviors play an important role in issues like antibiotic production, virulence or biofilm formation (Rutherford and Bassler, 2012).

Quorum sensing is a good example of a mechanism by which bacteria communicate through sensing the presence, in the extracellular milieu, of diffusible signaling molecules called autoinducers. The extracellular concentration of these autoinducers is higher for higher cell densities allowing individual bacteria to infer cell density and give an adequate response at the

level group (Waters and Bassler, 2005; Xavier and Bassler, 2003). There are a variety of autoinducers that play a role in communication between bacteria of a given species (Waters and Bassler, 2005). However, there are also an autoinducer playing a role in interspecies communication. This is the case of AI-2, a signal molecule associated with quorum sensing signaling between different species like *E.coli* and *S.enterica*. The gene enconding this universal signal molecule is *luxS* and without it there is no production of AI-2 (Xavier and Bassler, 2003).

Recently, other mechanisms for bacterial communication have been discovered. They are based on direct cell-cell contact, not on diffusible molecules (Aoki et al., 2005; Dubey and Ben-Yehuda, 2011; Wireman and Dworkin, 1977). These contact-dependent signaling systems also relies on high population densities since the higher number of cells the higher the chance of direct physical contact and subsequent signaling. Lately, this type of bacterial communication has been vastly investigated with novel mechanisms being discovered (Aoki et al., 2005; Gorby et al., 2006). *Myxococcus xanthus* is a Gram-negative bacteria that undergoes an multicellular development when in a nutrient limiting scenario (Wireman and Dworkin, 1977). Through cell contact mediated by a surface protein, *M. xanthus* cells communicate and aggregate to originate a multicellular form (Wireman and Dworkin, 1977). This contact-dependent process is called C-signaling and the surface protein associated is coded by *csgA* gene (Kim and Kaiser, 1990). Through this contact-dependent signaling and subsequent multicellular behavior, *M. xanthus* are able to predate other bacteria overcoming nutrient competition (Kaiser, 2008). Besides C-signalling in *M.xhantus*, there are reports of a contact-dependent signaling mechanism essential to sporulation in Gram-positive *Bacillus subtilis* also in nutrient-limiting environments (Feucht et al., 2003).

The cross-talk between cells through bridges formed by intercellular nanotubes is another example of direct contact communication discovered also in *B. subtilis* (Dubey and Ben-Yehuda, 2011). Through these nanotubes, bacterial cells are able to exchange cytoplasmic contents including antibiotic resistance proteins which lead to a non-hereditary resistance phenotype acquisition by neighbor cells. However Dubey and Ben-Yehuda also observed the transference of non-conjugative plasmids which makes this mechanism a way of acquiring hereditary traits. Additionally, intercellular exchanges also occur between bacteria from different species like *S. aureus* and *E. coli,* at an interspecies level (Dubey and Ben-Yehuda, 2011).

All the previous examples of contact-dependent mechanisms imply the exchange of contents beneficial to neighbor cells or rely on cooperative and multicellular behaviors. However, the contact-dependent growth inhibition (CDI) system uses direct cell contact to abolish the growth

of other bacteria (Aoki et al., 2005). Certain strains of *E.coli* harbor the *cdiBAI* gene cluster and thus are able to deliver a toxin into competing neighbor cells causing their growth to arrest. The *cdiB* gene encodes a β-barrel protein that allows the exportation of CdiA through the outer membrane. When cell contact occurs, CdiA interacts with the receptor of the target cell and is cleaved leaving its C-terminal toxin domain (CdiA-CT) to be exported across the outer membrane of the target cell into the cytoplasm (Aoki et al., 2005; Ruhe et al., 2013). Once inside the cell, the toxin disrupts the proton motive force leading to a decrease of ATP levels that subsequently originate the growth arrest of target cells (Aoki et al., 2009). However, CDI$^+$ bacteria prevent their own growth inhibition by producing an immunity protein. The *cdiI* gene encodes an anti-toxin protein that blocks the effect of CdiA-CT assuring that only CDI$^-$ cells are inhibited (Fig.1).
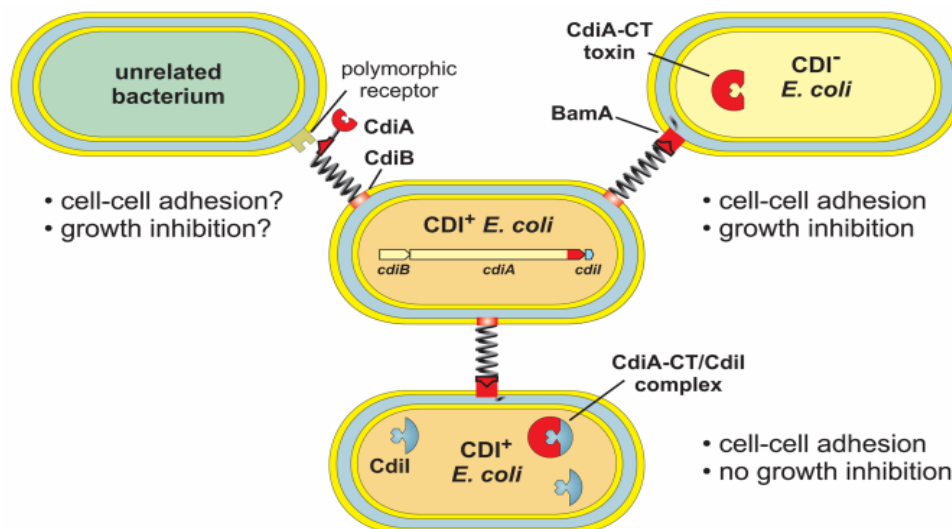

Figure 1. Contact-dependent growth inhibition (CDI) system in *E.coli.* Adapted from Ruhe *et al.*, 2013.

Since the early years of microbial investigation many studies focused on kinetics of bacterial growth, mainly on the identification and characterization of distinct growth phases (Ledingham and Penfold, 1914; Monod, 1949; Penfold, 1914; Winslow and Walker, 1939). Monod described six different growth phases and associated each one to a specific growth rate (Fig. 2). After inoculation the first phase of bacterial growth is the lag phase (1). Monod characterized this phase by a null growth rate. Following the lag phase, there is the acceleration phase (2) characterized by a growth rate increase. When the growth rate are

constant bacteria are in the exponential phase (3). When nutrient availability starts to decrease, there is a decline of the growth rate, specific of the retardation phase (4). After that, in stationary phase (5) the growth rate turns again to be null and then negative (6) (Monod, 1949).
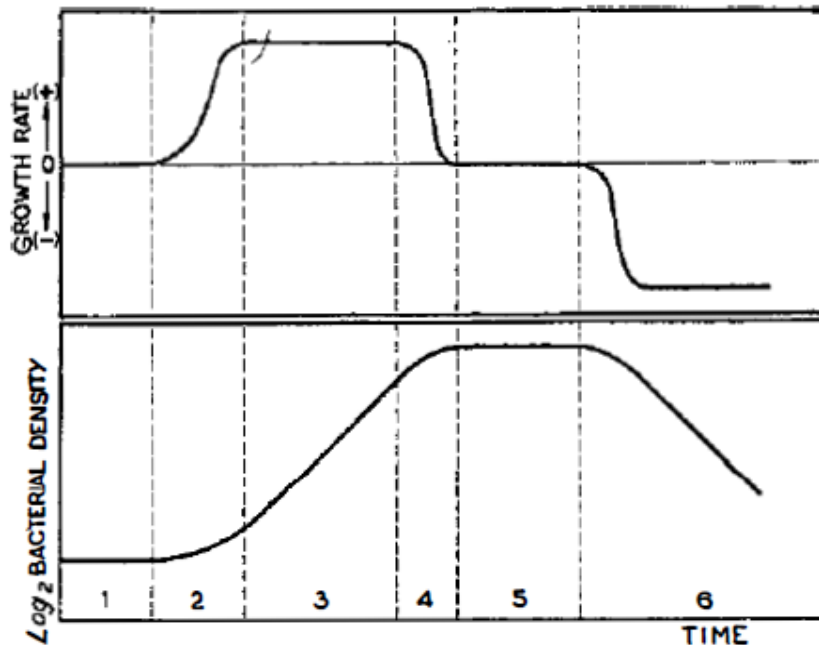

Figure 2. Association of bacterial growth phases with variations in the growth rate. Adapted from Monod, 1949.

All of the growth phases have been investigated throughout the years with an emphasis for the exponential phase followed by the stationary phase (Kolter et al., 1993; Lam et al., 2009; Zambrano and Kolter, 1996). However lag phase have been the most non-consensual growth phase in scientific community. While Penfold, in 1914, claims that lag phase is a latent period which duration is strongly influenced by temperature or inoculum size (Penfold, 1914), Monod, in 1949, states that lag phase is controlled by unknown mechanisms (Monod, 1949). Until today there is no enough strong evidence to end this discussion. However, advancements in technology have been providing scientists the necessary tools to enlighten scientific community about this matter (Rolfe et al., 2012). Rolfe *et al.* (2012) focused on the physiological and regulatory mechanisms taking place in the lag phase of *S. enterica* and concluded that a transient metal accumulation is an important process necessary to bacteria to proceed to exponential phase (Rolfe et al., 2012).

In a previous work it was shown that bacteria seem to have a growth advantage in high density competitions comparatively to low density ones (Nunes, 2012). In fact, 10^2 bacteria would perform more generations in the first hours of a 10^2:10^7 competition than 10^2 would do without competitors. This surprising result lead us to think that the 10^2 bacteria could somehow sense the high density competing bacteria and give a competitive response that is an initial rapid growth in order to anticipate the forthcoming nutrient competition. In addition Nunes (2012) also showed that such growth advantage only occurred when both populations were in the same physical space foreseeing some kind of direct cell contact communication. Knowing the existence of this association between high bacterial densities and higher growth rates the main objective of this thesis is to better understand this phenomenon by confirming the need of direct cell contact or proximity and trying to access the gene or genes involved in the process. For that, we studied 56 *E.coli* OMP mutants predicted by a bioinformatic tool (Zhou et al., 2008). Candidate genes would then be further investigated and restored in respective mutants to confirm their role in this contact-dependent communication phenomenon by a successful phenotype rescue.

MATERIAL AND METHODS

Bacterial strains

For the screening of Outer Membrane Protein (OMP) encoding genes (see Table 3) we used strains from the Keio Collection, a collection of systematic single-gene knock-out mutants (Baba et al., 2006). *ΔluxS* strain was also obtained from this collection. The parental strain of this collection is the *Escherichia coli* K12 strain BW25113. As reference isogenic strains, we used the JW0334 (*ΔlacY*) and JW3993 (*ΔmalF*) both with no apparent fitness cost. Another laboratory strain *E.coli* K12 MG1655 was used to obtain a spontaneous nalidixic-acid resistant mutant by plating an overnight grown culture (37 $^{\circ}$C, 170 rpm) in LB-agar supplemented with nalidixic-acid (40ug/ml) followed by overnight incubation at 37 $^{\circ}$C. A spontaneous mutant colony was then streaked in a new agar plate and a liquid stock was made and stored at -70 $^{\circ}$C. *Salmonella enterica* serovar Typhimurium LT2 and the firmicute *Staphylococcus aureus* NCTC 8325-4 were also used in this work.

Bacterial growth experiments

All bacterial growth experiments were performed using cultures grown overnight at 37°C with agitation (170 rpm) in 10 mL of Luria-Broth rich medium (LB). All serial dilutions were made in MgSO$_4$ 10^{-2}M. Low and high density (competition) experiments were assayed as follows:

I. Low density

For low density assays an aliquot of 100 µl of a 1:10^6 dilution of an overnight grown culture of the focal strain was used to inoculate 10 mL of LB.

II. High density (competition)

For high density assays an aliquot of 100 µl of a 1:10^6 dilution of an overnight grown culture of the focal strain was used to inoculate 10 mL of LB also inoculated with 100 µl of 1:10 dilution of an overnight grown culture of a reference strain.

In both cases an aliquot of 100 µl of the 1:10^6 dilution was also plated in LB-agar (LA) supplemented with proper antibiotics. The respective colony forming units (CFU) would then

be used to estimate the real number of bacteria at initial time of growth experiments, i.e. at the hour 0, t=0, and subsequently estimate the number of generations performed in a given time, t=T.

Both low and high density growth assays (three or five times replicated) occurred for 3.5 hours at 37 °C with agitation (170 rpm) and 100 µl of the adequate dilutions were then plated in LB-agar (LA) 1.5 % supplemented with antibiotic. Plates were incubated overnight at 37 °C.

Bacterial growth experiments on Tetrazolium-maltose medium

An initial pre-inoculum mixture was prepared by adding 100 µl of an overnight grown culture of the mutant strain and 100 µl of an overnight grown culture of the reference strain to an Eppendorf with 800 µl of $MgSO_4$ 10^{-2} M.

I.   Low density

For low density assays an aliquot of 100 µl of a $1:10^5$ dilution of the pre-inoculum mixture was used to inoculate 10 mL of LB.

II.   High density (competition)

For high density assays an aliquot of 100 µl of the pre-inoculum mixture was used to inoculate 10 mL of LB.

Both low and high density growth assays (three or five times replicated) occurred for 3.5 hours at 37 °C with agitation (170 rpm) and 100 µl of the adequate dilutions were then plated in TM (Tetrazolium-Maltose) medium. Plates were incubated overnight at 37 °C.

Calculation of number of generations and relative fitness

Let N(0) be the number of CFU at time t=0 and N(T) the number of CFU at t=T. The number of bacterial generations occurred in t hours was calculated as follows:

$$\text{No. generations} = \text{Log}_2[N(T)/N(0)]$$

The relative fitness of a given strain was calculated according to (Lenski, 1991) being the ratio between the number of generations performed by that strain and the number of generations performed by another strain.

Supernatant effect experiments

In order to test for the presence of a hypothetic signaling molecule released by the competitor strain we performed growth experiments in supernatants.

Three tubes with 10 mL of LB each were inoculated with 10^6 CFU/mL (100 µl of 1:10 dilution) of an overnight grown culture of a reference strain. The tubes were then incubated with agitation (170 rpm) at 37 ºC for 2.5, 3 and 4 hours. After that cultures were centrifuged at 4000 rpm for 20 minutes and each supernatant was filtrated to a new tube using a sterile syringe filter with a 0.2 µm polyethersulfone membrane. The resulting filtrated medium of each tube was then inoculated with about 10^2 CFU (100 µl of a $1:10^6$ dilution of an overnight grown culture of the focal strain). For comparison, low and high densities bacterial growth experiments (see above) were performed using the same strains.

Growth of bacterial macrocolonies for cellulose detection

Cells were grown overnight in liquid LB medium with agitation (170 rpm) at 37 ºC. A total of 5 µL of the overnight culture were spotted on LB agar plates supplemented with Calcofluor (100µg/mL). Plates with macrocolonies were incubated for 48h at 37ºC. Cellulose was detected by fluorescence of colonies on Calcofluor plates under 312-nm UV light.

Phenotype restoration (Plasmid transformation)

Gene amplification

The *bcsC* gene was amplified by Polymerase Chain Reaction (PCR) using primers as indicated below. PCR mixture reaction, for a final volume of 50 µl, consisted of 2.5 µl of bacterial DNA, 2 µl of both forward and reverse primers (10 µM), 18.5 µl of miliQ water and 25 µl of Phusion High-Fidelity PCR Master Mix with HF buffer. PCR amplification conditions were as follows:

95ºC for 2 min; 30 cycles of 98ºC for 5 sec, 54 ºC for 20 sec and 72 ºC for 90 sec; a final extension step of 7 min followed by storage at 4 ºC for infinity. 2 µl of PCR product were analyzed on an agarose gel electrophoresis (0.8 % agarose) to confirm product amplification. The remaining PCR product was then purified using Zymoclean Gel DNA Recovery Kit.

Table 1: Primers used for bcsC amplification by PCR

Gene	Primer name	Sequence
bcsC	bcsC-Fw-542	5´- GATCAACACCGTTTCCGCTTC –3'
	bcsC-Rev-3593	5'- AGATTGCCGCCCGAGTATAC–3'

Cloning on pJET vector and transformation of bacterial cells

After purified, all of PCR product was ligated to pJet 1.2/blunt cloning vector. The ligation mix consisted of 10 µl of 5X Reaction Buffer, 8 µl of gel recovered PCR product, 1 µl of Vector (50 ng/ µl) and 1 µl of T4 DNA ligase. The mix was left at room temperature for 30 minutes. After that, 5 µl of ligation mix were used to transform competent *E.coli* DH5α cells as follows: 200 µl of competent cells were mixed with 5 µl of ligation mix and the resulting mix was placed on ice for 30 minutes followed by a heat-shock step at 42ºC for 1 minute and 15 seconds. Then cells were placed back on ice for 2 minutes and 800 µl of LB were added, followed by incubation with slow agitation (50 rpm) for 1 hour at 37ºC. Cells were plated on LA supplemented with ampicillin (100 µg/ml) and incubated overnight at 37ºC.

The day after, a few recombinant colonies were isolated and purified before being used to inoculate 10 ml LB and be incubated at 37 ºC overnight for posterior plasmid extraction. Using QIAGEN Plasmid Mini Kit, plasmid was extracted and confirmed to be the recombinant plasmid containing the *bcsC* gene, by means of restriction analysis.

Subcloning on pUC19 and posterior phenotype restoration using pACD02

In order to subclone *bcsC* gene into pUC19 vector, the recombinant plasmid was digested using the restriction enzymes *Xho*I/*Xba*I. The resulting digestion was analyzed in a 0.8% agarose gel electrophoresis and the DNA fragment of interest (which englobes the *bcsC* gene) was recovered from gel using the Zymoclean Gel DNA Recovery Kit. The pUC19 vector was also digested with *Sal*I/*Xba*I to allow compatibility between vector and insert's overhangs. The ligation mix consisted of: 1 µl (about 100 ng) of pUC19 vector, 6 µl (about 450 ng) of insert (DNA fragment with the *bcsC* gene), 1.5 µl of ligase buffer (10 x), 1 µl of T4 DNA ligase and 5.5 µl of miliQ water up to a final volume of 15 µl. Ligation mix was incubated overnight at room temperature. 200 µl of competent cells of *E.coli* DH5α were then transformed as described

above using 7.5 µl of ligation mix. Transformed cells were confirmed to have the right molecular construction (i.e. the *bcsC* gene cloned on pUC19) by restriction analysis of extracted plasmid. The resulting recombinant plasmid is henceforth named pACD02. Finally competent Δ*bcsC* *E.coli* cells were transformed with pUC19 (as a control) and with pACD02 (in order to become *bcsC*-restored).

Phenotype restoration (P1 Transduction)

P1 transduction is a well-known method to transfer alleles or bacterial chromosome regions from one bacterium to another using the generalized transducer bacteriophage P1. When infecting bacteria, P1 is capable of capturing DNA fragments of approximately 100 kb from that bacterial strain (the donor strain). A phage lysate is formed and then it is used to infect another strain (the recipient strain). DNA fragments from the donor strain will now integrate in the chromosome of the recipient strain by homologous recombination.

Removal of antibiotic-resistance gene cassette

The helper plasmid pCP20 ($Cm^R Amp^R$) from Wanner and Datsenko is used to eliminate the kanamycin-resistance gene cassette present in *E.coli* K12 strains from Keio collection, such as strain *E.coli* K12 Δ*bcsC*. To do so 200 µl of competent cells of Δ*bcsC* strain were transformed with 1 µl of the helper plasmid pCP20 ($Cm^R Amp^R$) in the same way as previously described here with the exception that this time incubation steps occurred at 30ºC instead of 37 ºC, once pCP20 has temperature-sensitive origin of replication. Transformed colonies were then streaked onto LA and grown overnight at 42ºC in order to lose pCP20 plasmid and thus resistance to chloramphenicol and to ampicillin. Grown colonies were confirmed by colony PCR to lack the Kanamycin-resistance gene cassette.

Preparation of P1 lysate

The *E.coli* K12 Δ*dctA* strain from Keio collection was chosen to be the donor strain as *dctA* is an upstream close neighbor gene of *bcsC* allowing P1 to encapsidate both Kanamycin-resistance gene cassette and the wild type allele of *bcsC.* This strain was grown overnight at 37º C in LB. 100 µl of the overnight grown culture were used to inoculate 5 ml of LB supplemented with 0.2% glucose and 5 mM $CaCl_2$, which was then incubated at 37ºC for 1 hour with agitation (170 rpm). After that 100 µl of a P1 lysate obtained from a WT strain were added to tube following a 3 hour incubation step at 37 ºC with agitation (170 rpm). The culture was visually checked until cells lyse. Then 100 µl of chloroform were added to the tube that was centrifuged at 4500g for 10 minutes to pellet debris. Supernatant containing P1 transducing particles (P1 lysate) was treated with more 100 µl of chloroform and then carefully transferred to a sterile glass tube which was stored at 4ºC.

P1 transduction

Recipient strain (Δ*bcsC*) was grown overnight at 30 ºC in LB supplemented with 10mM MgSO$_4$ and 5 mM CaCl$_2$. 100 µl of recipient cells were mixed with 100 µl of a 1:10 dilution of P1 lysate (from strain *dctA*-) and incubated at 37 ºC for 20 minutes without agitation. 400 µl of LB supplemented with 50 mM citrate were added following 1 hour and 30 minutes of incubation at the very same conditions. The tube was centrifuged for 2 minutes, the supernatant discarded and pellet was resuspended in 400 µl of 0.1M Citrate buffer (pH 5.5). Cells were plated on selective media and incubated overnight at 37ºC. For two days, colonies were streaked on selective media for purification to obtain a single, phage-free colony.

E.coli K12 MG1655 *bcsC* knockout

In order to knockout the *bcsC* gene directly from *E.coli* K12 MG1655 Nal[R] we used the λ red system used by Wanner and Datsenko to disrupt chromosomal genes in *E.coli* K12 (Datsenko and Wanner, 2000). To amplify a DNA sequence constituted by a chloramphenicol-resistance cassette flanked by FRT (FLP recognition target) sites from the pKD3 plasmid, primers were designed as described elsewhere (Datsenko and Wanner, 2000); (Baba et al., 2006) and listed in the table below.

Table 2. Wanner primers

Target Sequence	Primer name	Sequence
Cloramphenicol resitance cassette	bcsC_fw_cmpR	5' **TTCCGCTTCTCGACAAAAGGTGAGTTATTACCTGACTGGGGCCAGGAATG**ATTCCGGGGATCCGTCGACC 3'
	bcsC_rev_cmpR	5' **CGGCATTAAGAGAGGCGCTATCTGAAAACTTACCAGTCGGCGTAAGGTA**TTGTAGGCTGGAGCTGCTTCG 3'

PCR reaction mix consisted of 2.5 µl of both forward and reverse primers, 1 µl of 1:1 or 1:10 DNA template (pKD3 plasmid), 19 µl of nuclease-free water and 25 µl of 2X Phusion Master Mix in a final volume of 50 µl. PCR amplification conditions were as follows: 98ºC for 30 seconds; 30 cycles of 98 ºC for 10 sec, 55 ºC for 20 sec and 72 ºC for 60 sec; a final extension step of 7 min followed by storage at 4 ºC for infinity. Amplification was confirmed by analyzing 5 µl of PCR product on an agarose gel electrophoresis (0.8 % agarose). The amplified DNA was purified and digested with the restriction enzyme *Dpn*I in the following conditions: 0.5 µl of *Dpn*I were mixed with 5 µl of 10X *Dpn*I buffer and 45 µl of PCR product and the mix was incubated overnight at 37ºC. Using Nucleospin Gel and PCR clean-up kit, we were able to

purify the DNA after enzymatic digestion. Nanodrop measuring pointed to a final DNA concentration value of 110 ng/µl.

For electroporation of the purified DNA into recipient cells, 2 ml of *E.coli* MG1655 Nal^R culture grown overnight at 30ºC in LB supplemented with ampicillin (100 µg/ml) were added to 200 ml of SOB (Super Optimal Broth) medium supplemented with 200 µl of 1M arabinose and 200 µl ampicillin (100 µg/ml) in a flask. Cells were grown at 30 ºC until absorbance values at OD at 600 nm were between 0.6 and 0.8. After this the culture was divided into 4 tubes of 50 ml each that were then centrifuged at 4000 rpm at 4 ºC for 20 minutes. The supernatant was discarded and the pellet was lightly resuspended in 40 ml of 10% glycerol. The previous two steps were repeated two more times with the last resuspension being made in the remaining 10% glycerol after supernatant discard.

50 µl of electrocompetent cells were mixed with 1.5 µl of purified DNA (165 ng/µl) and kept in ice. This mix was then added to a ice-cooled electroporation sterile cuvette and cells were electroporated at the following conditions: 200 Ω, 25 µF and 2.5 Kvolts. Immediately after electroporation 1 ml of SOC medium was added to the cuvette which was incubated for 2 hours at 37ºC without agitation. After this period, 500 µl of the sample were centrifuged for 2 minutes at 10000 rpm. 400 µl of supernatant were removed and the remaining 100 µl were plated in LA supplemented with Cm (50 µg/ml). Plates were then incubated overnight at 37 ºC and colony PCR confirmed the *bcsC* gene deletion.

Statistical analysis

The data presented here were analyzed using Microsoft Office Excel 2013 and IBM SPSS Statistics 22. One-way ANOVA and Student t-test were performed to evaluate significance. p-values were adjusted using the Holm-Bonferroni correction for multiple comparisons, when indicated. An adjusted p-value <0.05 was used as the cut-off for statistical significance. ns Not significant; *p-value <0.05 and **p-value <0.01.

RESULTS

High density-dependent growth advantage occurs by interaction between different Gram-negative species

As previously described, several strains of *E. coli* bacterial cells grow faster at high densities than at low densities (Nunes, 2012). This phenomenon has been observed under different experimental designs but it is clearer when one compares the growth of 10^2 bacteria alone in LB rich media (thus assaying growth at a very low density) with the growth of the same (isogenic) 10^2 bacteria competing with other 10^7 bacteria (thus assaying growth at a high density). In this thesis work we mainly use mutants from the Keio collection, so we started by confirming that one of the strains of this collection behaves as expected. Henceforth, and unless otherwise mentioned, we used the Δ*malF* mutant of this collection (which is unable to use maltose as carbon source), as our reference strain, therefore mentioned as *E.coli* WT (for *wild-type*). Indeed, and as expected, 10^2 cells of this *E. coli* WT strain completed 5.77 ± 0.73 generations at low densities (growing alone) during 3.5 hours but completed 7.21 ± 0.33 generations, during the same time, when competing with 10^7 cells of *E.coli* K12 Nal^R (p = 0.0002, Student t-test paired) (Fig. 3). Interestingly, this behavior may occur between two different species as well, in particular with the enterobacterial species, *Salmonella enterica* serovar Typhimurium and *Escherichia coli*. Indeed 10^2 cells of *S. enterica* alone (low density) completed 6.32 ± 0.09 generations, but achieved 7.03 ± 0.18 generations when in competition with 10^7 *E. coli* Nal^R cells (p = 0.005, Student t-test paired) (Figure 3). However, the firmicute *Staphylococcus aureus* NCTC 8325-4 failed to achieve this growth advantage when competing with 10^7 cells of *E.coli* (p = 0.401, Student t-test paired) (Figure 3), a result that indicates this phenomenon could be specific of the major group of proteobacteria.
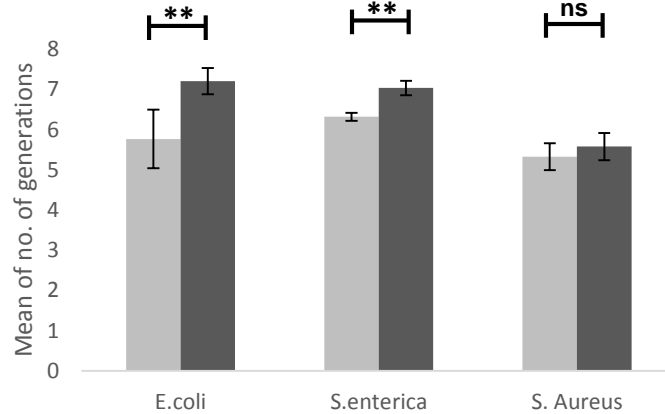
Fig.3. Growth of 10^2 cells of _E.coli, S.enterica_ or _S.aureus_ at low or high density (alone or competing with 10^7 E.coli,_ respectively). Mean (5 replicates) of number of generations performed in 3.5 hours by 10^2 cells of indicated species growing alone (light grey bars) or growing with 10^7 _E.coli_ (dark grey bars). Error bars represent twice the standard deviation of the mean, * stands for a p-value < 0.05; ** for p-value <0.01; ns for not significant.

High density-dependent growth advantage depends on cell-cell contact

We then tested whether AI-2 is involved in the phenomenon observed in Fig. 3. If AI-2 is involved, then 10^7 competing cells would produce the Gram-negative autoinducer AI-2 and the 10^2 cells would read such signal and respond to it with a higher growth rate. To test this hypothesis, we compared the growth rate of 10^2 cells alone _versus_ in competition with 10^7 cells of the Keio mutant lacking the _luxS_ gene, thus incapable of producing the signal molecule AI-2. However, our results showed that the growth advantage in high densities still persists even when the competing bacteria in high density lack the _luxS_ gene. Indeed, 10^2 _E.coli_ WT _ΔlacY_ cells completed 6.19 ± 0.08 generations alone in LB but when in competition with 10^7 _E.coli ΔluxS_ cells or with 10^7 _E.coli_ Nal^R, they completed 7.10 ± 0.18 and 7.09 ± 0.20 generations, respectively (Fig. 4A). One-way ANOVA analysis with post hoc Tukey's test indicates that are significant differences between the three groups ($n = 3$, d.f. = 8, $p = 0.013$, one-way ANOVA). Those differences exist between bacteria growing alone and bacteria growing with 10^7 _E.coli ΔluxS_ cells ($p = 0.020$, Tukey test) or with 10^7 _E.coli_ Nal^R ($p = 0.021$, Tukey test). On the other side, there is no significant difference between bacteria growing with 10^7 _E.coli ΔluxS_ cells and bacteria growing with 10^7 _E.coli_ Nal^R cells ($p = 0.998$). This excludes the participation of the AI-2 as the sensing signal.

However other yet undiscovered signaling molecules could be responsible for this phenomenon. This lead us to test the effect of the supernatant of growing cultures of 10^7 _E.coli_ Nal^R cells for the presence of hypothetical diffusible molecules. We inoculated 10^2 _E.coli_ cells in fresh LB and in the supernatant of cultures of 10^7 _E.coli_ cells with different growing times (2.5, 3.0 and 4.0 hours), as well as in LB in competition with 10^7 cells. The 10^2 cells only have

a burst in their growth advantage when in the presence of the 10^7 cells; moreover, the growth rate in fresh LB and in any of the three supernatants was similar (Figure 4B). These results suggest that some type of contact-dependent signaling is involved in this phenomenon and henceforth it will be named contact-dependent growth advantage. However, these results do not exclude the possibility that communication occurs through the secretion of a very unstable metabolite.
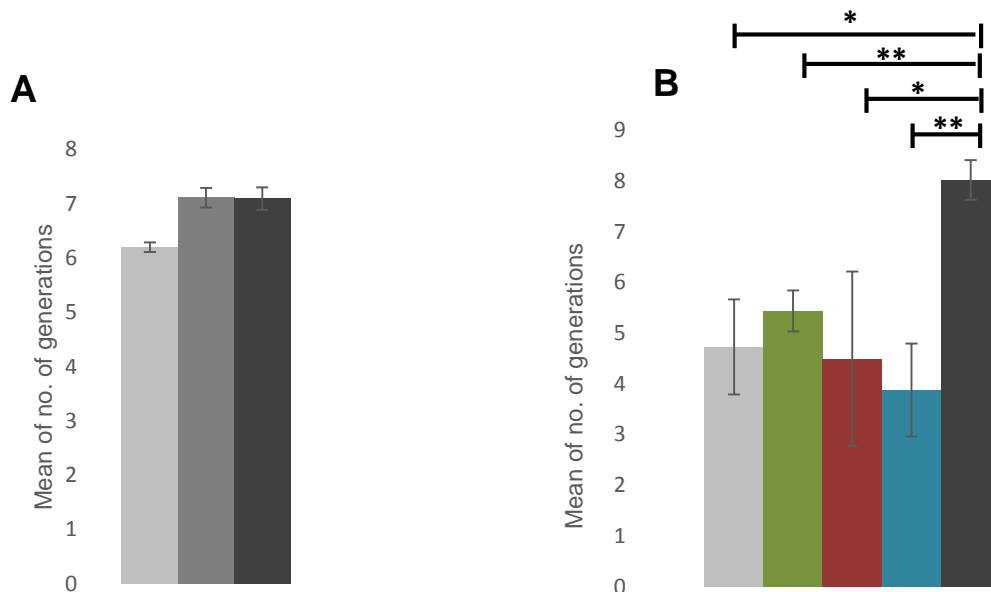

Fig.4. Exclusion of diffusible signaling factors. A. 3.5 hours growth of 10^2 cells of *E.coli* in LB (light grey bar), in LB with 10^7 *E.coli ΔluxS* cells (grey bar) and in LB with 10^7 *E.coli* WT cells (dark grey bar). **B.** 3.5 hours growth of 10^2 cells of *E.coli* in LB (light grey bar), in filtered supernatants of cultures of 10^7 *E.coli* WT cells grown for 2.5, 3 and 4 hours (green, red and blue bars, respectively) and in LB with 10^7 *E.coli* WT cells (dark grey bar). Error bars represent twice the standard deviation of the mean, * stands for a p-value < 0.05; ** for p-value <0.01, Student t-test (Bonferroni correction for multiple comparisons).

Screening of OMP encoding genes

Given that this growth advantage requires direct contact between cells for this signaling to occur, we tested the participation of one or more proteins of the outer membrane of proteobacteria. Through the bioinformatic tool *LocateP* v2 (Zhou et al., 2008) we listed 56 predicted Gram-negative OMP (see Table 3) and tested them for the contact-dependent growth advantage phenomenon. Using the Tetrazolium-maltose medium system we were able

to compare simultaneously the growth of each OMP mutant and the *E.coli* K12 WT strain ($\Delta malF$) through 1:1 competitions at the following densities (mut:WT) of 10^2:10^2 (i.e. at low density) or 10^7:10^7 (high density).With this method we measured the fitness of each mutant relative to that of the reference wild-type strain, both at low and high densities. Then we calculate the difference of these fitness values (i.e., fitness at high density minus fitness at low density. If this difference is negative, it means that the expected increase of the growth rate of the wild-type strain is not accompanied by an equivalent increase of that of the mutant. This means that at high densities the $\Delta malF$ (wild-type) strain experienced increased growth rate associated to higher density while the OMP mutant did not behave in the same way. Such difference would indicate an impairment in the communication system at high density and thus being considered a good candidate for further investigation. Three mutants indeed showed a lower relative fitness at high density than at low density. They were the mutants lacking the *pqqL*, *yncD* and *bcsC* genes (Table 3).

Table 3. OMP screening

OMP mutants	Relative fitness at low density	Relative fitness at high density	Difference of fitnesses high minus low	Student t-test
yehA	0,91	0,63	-0,28	0,505
yncE	0,77	0,93	0,16	0,090
ompA	0,97	0,99	0,02	0,924
fiu	0,88	0,95	0,07	0,836
yhcP	1,23	0,98	-0,24	0,541
ydiY	1,54	1,64	0,10	0,863
rcsF	1,52	1,64	0,13	0,797
ydeS	0,41	0,84	0,42	0,001
yhcD	0,56	0,55	-0,01	0,938
csgG	0,97	1,02	0,05	0,928
yraP	3,18	2,24	-0,93	0,056
yftM	1,59	1,62	0,03	0,891
spr	0,39	0,61	0,22	0,335
yftN	2,75	3,70	0,96	0,162
mdtA	1,24	1,04	-0,20	0,525
rseB	0,60	0,62	0,02	0,957
mltD	1,83	1,52	-0,32	0,249
ycbF	1,29	1,49	0,20	0,467
ycbV	2,11	1,66	-0,45	0,054
ybgO	1,21	1,32	0,10	0,605
mltC	0,74	0,90	0,16	0,320
tolC	1,35	0,98	-0,37	0,488
ybgQ	1,54	2,09	0,54	0,312
yfaZ	0,81	1,44	0,63	0,209

pqqL	**2,84**	**1,78**	**-1,07**	**0,031**
yfaP	1,49	2,12	0,62	0,140
yiaT	2,90	1,60	-1,30	0,238
ybhG	0,90	1,20	0,30	0,228
fhuE	0,95	1,41	0,46	0,335
ycgK	1,78	2,24	0,46	0,728
ydhJ	1,40	1,08	-0,32	0,435
nfrA	1,57	1,49	-0,08	0,915
yncD	**1,22**	**0,83**	**-0,38**	**0,043**
hofQ	1,04	1,39	0,35	0,246
htrG	0,85	0,91	0,07	0,389
ymcA	0,62	0,74	0,12	0,708
yibG	2,74	1,40	-1,34	0,462
ygjJ	1,52	1,76	0,23	0,681
yicH	0,91	0,64	-0,27	0,572
yjcP	0,93	0,80	-0,13	0,348
yjbH	1,64	1,01	-0,63	0,290
hemX	0,60	0,41	-0,19	0,435
uidC	0,44	0,53	0,10	0,524
ymbA	0,79	0,37	-0,41	0,461
yegX	0,77	0,58	-0,19	0,055
amiB	1,07	0,83	-0,24	0,348
emrK	0,64	0,90	0,25	0,332
envC	0,12	0,22	0,10	0,147
gspD	0,79	0,75	-0,04	0,419
bcsC	**0,59**	**0,29**	**-0,30**	**0,040**
acrE	1,09	0,98	-0,10	0,800
yjiK	0,28	0,45	0,17	0,018
ymcC	1,19	1,00	-0,19	0,506
acrA	0,61	0,47	-0,14	0,337
mdtE	0,78	0,99	0,21	0,316
aaeA	0,87	0,90	0,03	0,815

Δ*pqqL* strain has a relative fitness of 2.34 at low density and 1.78 at high density, a difference that is significant (n=3, p = 0.031, Student t-test). This suggests that the WT strain was more effective in sensing the high density competition than the mutant. Similarly, the relative fitness of the mutant Δ*yncD* at low and high density is 1.22 and 0.83, respectively, a difference that is significant (n=3, p = 0.043, Student t-test). A difference was also observed for the Δ*bcsC* strain: the relative fitness at low density was 0.59 and, at high density, was 0.29, a difference that is significant (n=3, p = 0.040). These three mutants show evidence of an impaired capability of

sensing high density competition. These three cases of OMP mutants are thus good candidates for further investigation.

Δ*bcsC* and Δ*pqqL E.coli* strains fail to exhibit high density-dependent growth advantage

To confirm that these three mutants are indeed unresponsive to high bacterial densities, we performed growth experiments of each of these 10^2 cells of the mutant alone (low density) and in competition with 10^7 cells of E.coli Nal[R]. The mutant *E.coli* Δ*pqqL* completed 5.45 ± 0.39 generations at low density and 5.78 ± 0.41 generations at high densities, a difference that is not significantly different (n=5, p=0.071, Student t-test). Similarly, *E.coli* Δ*bcsC* completed 4.65 ± 0.74 generations at low density and 5.01 ± 0.76 generations at high density, a difference that is not significant (n=5, p = 0.883, Student t-test paired). However, Δ*yncD* completed more generations at high density than at low densities: 7.37 ± 0.88 in low density and 9.01 ± 0.29 in high density, a difference that is significant (n=5, p = 0.041, Student t-test paired). For control, two different *E.coli* K12 strains were used, namely BW25113 and MG1655 which performed, both, significantly more generations at high density (Fig 5).
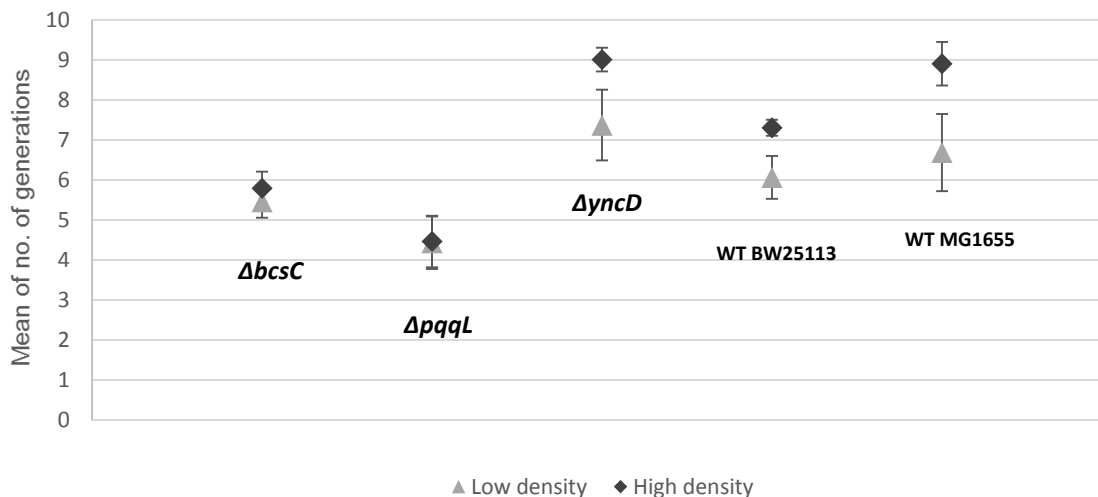

Fig.5. ΔbcsC and ΔpqqL E.coli strains fail to exhibit contact-dependent growth advantage. 3.5 hours growth of 10^2 cells of the represented *E.coli* strains in LB, i.e. in low density (light grey triangles) and in LB with 10^7 *E.coli* WT cells, i.e. in high density (dark grey diamonds). Error bars represent twice the standard deviation of the mean, * stands for a p-value < 0.05; ** for p-value <0.01, Student t-test paired.

Given these results we asked what would happen if the 10^7 cells have their *pqqL* and *bcsC* genes deleted. That is, how would these cells affect the growth rate of 10^2 wild-type cells. To

do that we simply inverted the strains in our growth experiments by comparing the growth of 10^2 WT cells in LB alone to the growth in LB with 10^7 competing $\Delta bcsC$, $\Delta pqqL$ or WT cells. As seen in Figure 6, when competing with the wild-type cells, the 10^2 cell population achieved 7.82 ± 0.31 generations instead of the 5.99 ± 0.40 generation they do growing alone. However, when competing with 10^7 cells of $\Delta pqqL$ strain, they still grow significantly more than they do alone but less than they do when competing with the WT strain (Fig 6A). A one-way ANOVA analysis was crucial to determine that the number of generations performed by bacteria depended on the genotype of the competing strain (n = 5, d.f. = 14, p = 0.004, one-way ANOVA). Post-hoc analysis confirmed that the 10^2 cell population performed significantly better only when competing with WT strain (p = 0.003, Tukey test).

Similarly, when the $\Delta bcsC$ strain is at high density, there are significant differences in the growth of 10^2 WT cells alone and the growth with 10^7 cells of $\Delta bcsC$ strain or with 10^7 WT cells (n = 5, d.f. = 14, p = 0.023, one-way ANOVA). Tukey test enlighten us about those differences and confirmed that only growing alone is different from growing with 10^7 WT cells (p = 0.026, Tukey test).
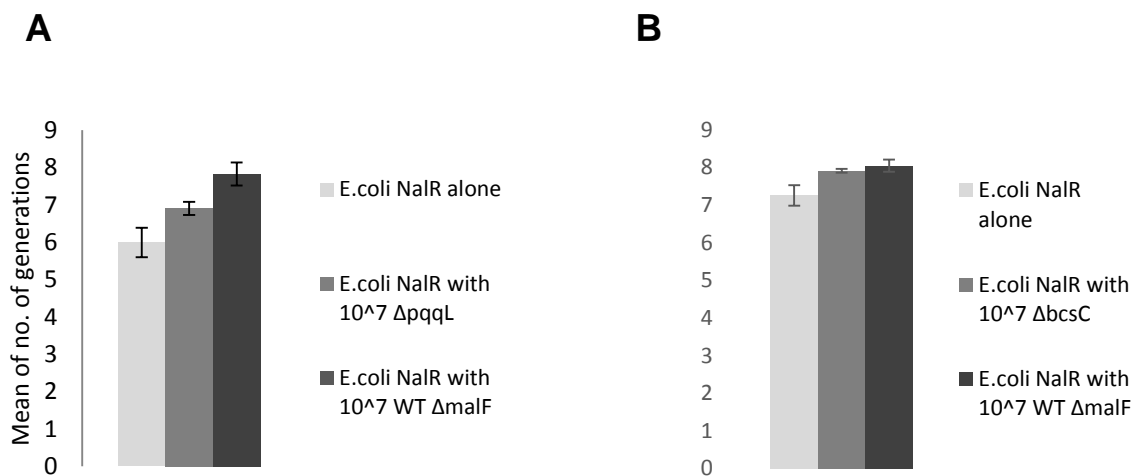
A   **B**

Fig.6. Effect of $\Delta pqqL$ and $\Delta bcsC$ competing cells on growth of 10^2 cells. **A.** Mean of number of generations performed in 3.5 hours by 10^2 cells of the *E.coli* NalR strain in LB alone (ligh grey bar), in LB with 10^7 *E.coli* $\Delta pqqL$ cells (grey bar) and in LB with 10^7 *E.coli* WT ΔmalF (dark grey bar). **B.** Mean of number of generations in 3.5 hours growth of 10^2 cells of the *E.coli* WT nalR strain in LB alone (ligh grey bar), in LB with 10^7 *E.coli* $\Delta bcsC$ cells (grey bar) and in LB with 10^7 *E.coli* WT ΔmalF (dark grey bar).). Error bars represent twice the standard deviation of the mean.

Replacement of *bcsC* in ΔbcsC strain failed to rescue the contact-dependent growth advantage phenotype

In order to test whether BcsC is a critical protein to the high density-dependent growth advantage phenotype we decided to rescue such phenotype by re-introducing the *bcsC* gene in the *E.coli ΔbcsC* mutant strain by two different methods.

First, we cloned the *bcsC* gene in the plasmid pUC19, giving origin to recombinant plasmid pACD02 (see Methods). In an attempt to check the effect of constitutive expression of *bcsC* in recombinant bacteria and knowing that *bcsC* plays a role in bacterial cellulose synthesis we evaluate, qualitatively, the production of cellulose on LB agar plates supplemented with Calcofluor (CF). In figure 7, we show that only the recombinant strain (and not the negative-control strains) was able to display a fluorescent phenotype, indicative of biosynthesis and secretion of cellulose.
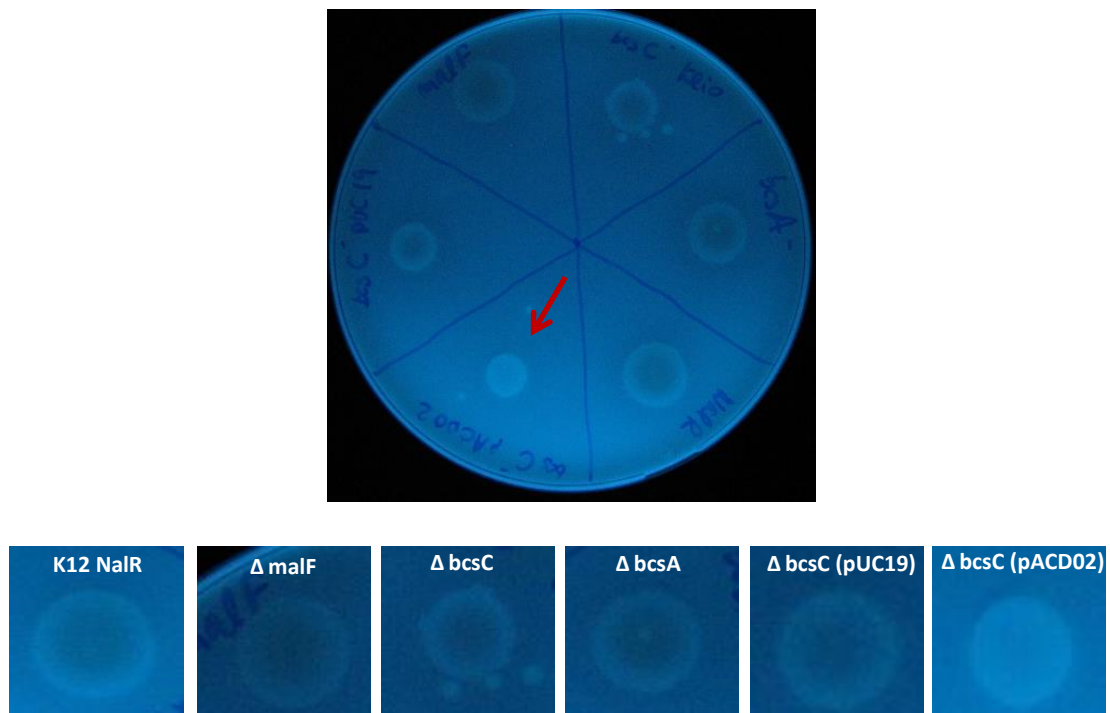

Fig.7. Cellulose biosynthesis on CF plates. Only the recombinant strain ΔbcsC + pACD02 displays a fluorescent phenotype (red arrow), indicative of cellulose biosynthesis.

However, with this method, we did not rescue the high density-dependent growth phenotype. Surprisingly, bacteria with the control vector pUC19 were also incapable of display such phenotype (discussed later).

The second method consisted on the site-directed replacement of *bcsC via* P1 transduction. We thus eliminated any mobile genetic element that could be interfering with the phenotype in

study. However, even in this case we did not rescue the phenotype. As seen in figure 8 10^2 cells of Δ*bcsC* strain performed 5.59 ± 0.66 generations growing alone and 6.08 ± 0.43 generations when competing with 10^7 WT cells (n=5, p = 0.064, Student t-test paired). Nonetheless the *bcsC*-restored strain presents a similar result: alone 10^2 cells of this strain performed 5.97 ± 0.83 generations growing alone and 6.12 ± 0.60 when competing (n=5, p = 0.584, Student t-test paired). On the other hand and as expected, the WT Δ*malF* strain performed 5.61 ± 0.91 generations growing alone but reached 7.12 ± 0.38 generations in competition with 10^7 WT Nal^R cells (n=5, p = 0.003, Student t-test paired).

Together, these results show that, although the restoration of *bcsC* gene *via* P1 transduction was successful (confirmed by PCR), it failed to rescue the contact-dependent growth advantage phenotype.
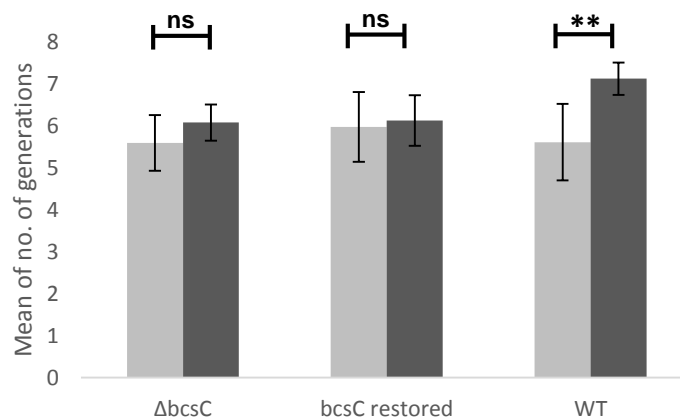

Fig.8. Restoration of *bcsC* does not rescue the contact-dependent growth advantage phenotype. 3.5 hours growth of 10^2 cells of *E.coli* Δ*bcsC*, *bcsC^restored* or WT in low or high density (alone or competing with 10^7 E.coli, respectively). Light grey bars represent the low density and dark grey bars represent the high density competition). Error bars represent twice the standard deviation of the mean, ** stands for p-value <0.01; ns for not significant.

Deletion of *bcsC* in the WT Nal^R strain did not abolish the contact-dependent growth advantage

Given the previous results we asked whether the rescue of the contact-dependent growth advantage phenotype could be being masked by an unknown mutation accumulated by the Keio collection Δ*bcsC* strain. Thus, we knocked out the *bcsC* gene from the *E.coli* WT Nal^R strain using the λ red system (Datsenko and Wanner, 2000). The results clearly show that the contact-dependent growth advantage still persists in *E.coli* Nal^R Δ*bcsC* strain.
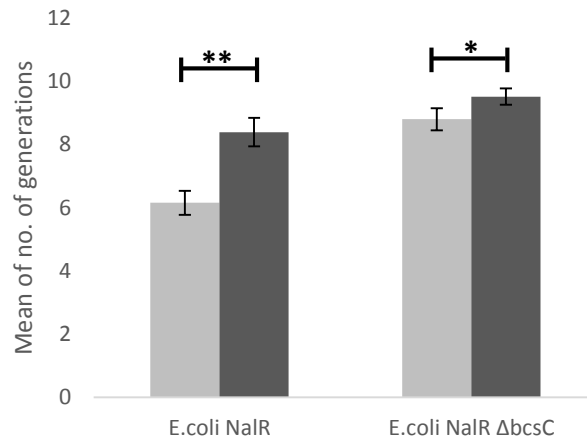
Fig.9. Growth of 10^2 cells of *E.coli* NalR or *E.coli* NalR ΔbcsC at low or high density (alone or competing with 10^7 *E.coli*, respectively). Mean (5 replicates) of number of generations performed in 3.5 hours by 10^2 cells of indicated strains growing alone (light grey bars) or growing with 10^7 *E.coli* (dark grey bars).). Error bars represent two times the standard deviation of the mean, * stands for a p-value < 0.05; ** for p-value <0.01.

As expected, the *E.coli* NalR strain performed 6.15 ± 0.38 generations at low density while at high density this strain reached 8.39 ± 0.45 generations (n = 5, p = 0.0005, Student t-test paired). Similarly and contrary to our expectation, the isogenic ΔbcsC strain also completed more generations at high density than at low ones achieving 9.51 ± 0.26 generations and 8.80 ± 0.34 generations, respectively (n =5, p = 0.0017, Student t-test paired). Altogether these results seem to exclude any role of *bcsC* gene in contact-dependent growth advantage phenomenon.

DISCUSSION

In this thesis, we show that a small population of *E. coli* grows faster when in the presence of a high-density population of *E. coli*. A small population of *S. enterica* also grows faster when in the presence of a high-density population of *E. coli*. From the ecological point of view, these results are paradoxical given that the high-density population is consuming nutrients, leaving less nutrients to the small population.

Interestingly, the firmicute *S. aureus* did not grow faster when in the presence of a high-density bacterial population (either of *S. aureus* (data not shown) or of *E. coli*). At this point it is unclear whether this phenomenon would be observed among other *Enterobacteriaceae* or even among the entire Gram negative major group. *Enterobacteriaceae* species are known to habitat the human gut, a habitat where large communities with hundreds of species co-exist and have to compete for nutrients and space (Walter and Ley, 2011). Thus, any mechanism to alert bacteria for the presence of competitors would be positively selected. Such communication would be translated through some molecular signaling that rush bacterial growth.

Most signaling mechanisms known involve the secretion and detection of diffusible molecules in the growth media (Miller and Bassler, 2001). However, supernatants resulting from high density cultures did not have a positive effect on the growth of a small population of *E. coli*. Additionally, our experiments ruled out any role to the autoinducer AI-2, given that, when the cells of the high-density population lacked the *luxS* gene, the focal population still was able to sense the competitors and complete more generations in their presence. Therefore, these diffusible molecules can be rejected as involved in the signaling for the growth promotion mechanism. However, we do not discard the possibility of excretion of very unstable metabolites that could be easily lost during the process of gathering supernatant.

Given the need of cell contact in this phenomenon, we considered the possibility that this signaling occurs by interaction between proteins of the outer membrane of *E. coli.* We thus listed 56 predicted outer membrane proteins of Gram-negative bacteria and worked with the corresponding knockout mutants of the Keio collection. An interesting mutant would be identified if it fulfills certain conditions using two different methods: (i) using the tetrazolium-maltose system; and (ii) growth experiments alone and with competitors. Using the tetrazolium-maltose system, a method that allowed us to analyze the relative fitness of each mutant at low and high densities (relative to a reference strain), we were able to detect three promising genes: *bcsC, yncD* and *pqqL*. The Keio mutants without each of these three genes have a lower relative fitness (relative to the wild-type strain) at higher densities than at lower densities. A lower fitness at high-density means that, for some reason, the mutants did not sense and/or

react to the high density population in the same way that the WT did. This could be due to the lack of the indicated genes but also due to an unknown effect caused by the different experimental conditions between this and the classic growth assays (see methods). Among these three mutants, the *yncD* Keio mutant completed more generations at high-densities than at low-densities, hence failing the second requirement (growth experiments). In conclusion, these results suggest a role for the *bcsC and pqqL* genes in the contact-dependent growth advantage signaling.

In a communication process, there is a sender and a receiver of the message. When two bacterial cells communicate by cell-cell contact, the molecule (e.g. a protein) that senses the contact may be the same or different from the molecule that signals the presence of the cell. Therefore, after showing that the *bcsC and pqqL* genes are important to sense other bacteria, we asked whether these genes are also signaling to the other cells. So we tested whether the absence of those genes in the competing population affects the growth advantage mechanism. We concluded that neither *bcsC* nor *pqqL* have an effect in such mechanism in this condition. Altogether these results suggest that the signal given by the competing population to the low density one depends on other proteins.

According to literature *bcsC* encodes a protein described as a cellulose synthase subunit spanning periplasm and outer membrane (Romling and Galperin, 2015). Included in the highly diverse *bcs* operon, *bcsC* is necessary for maximum cellulose exportation across the outer membrane of many bacteria (even in closely related species) (Romling and Galperin, 2015). Bacterial cellulose biosynthesis may occur in many circumstances in bacterial world but this process is more known by its role in mechanisms like biofilm formation and thus has been proven an important step in host colonization by pathogenic bacteria (Solano et al., 2002). Since biofilm formation implicates cell-cell interactions to achieve a final multicellular structure, cellulose biosynthesis could be an intermediate process leading to cell-cell interactions and the signaling that would confer contact-dependent growth advantage. To confirm the role of *bcsC* in this new contact-dependent communication system we tried to complement the mutant Δ*bcsC* by transforming cells with the recombinant plasmid pACD02 (i.e. constitutively expressing *bcsC*). However, recombinant cells failed to complete more generations at high density competitions thus indicating an unsuccessful phenotype rescue. To check for the effect of constitutive expression of *bcsC* we qualitatively evaluate cellulose biosynthesis in the recombinant strain. Our results show that only the latter but not any of the negative controls displayed a positive phenotype for cellulose production in CF plates. Thus, an exaggerated cellulose biosynthesis could be interfering with the contact-dependent signaling mechanism here described while an endogenous *bcsC* expression would be the key for this signaling to occur. All previous studies have also proved that the laboratory *E. coli* K12 strain is incapable

of synthesizing cellulose (Zogaj et al., 2001). More recently it was shown that a point mutation in the recently discovered *bcsQ* gene, upstream of *bcsC*, is responsible for this failure in the polysaccharide synthesis (Serra et al., 2013). Interestingly, our results contradict the literature: we clearly show that *E. coli* synthesize cellulose and prove that the constitutively expression of *bcsC* is a necessary and sufficient condition for such ability. However, even with the control vector pUC19, we were not able to rescue the contact-dependent growth advantage phenotype. Probably, the reason is that this method implies a fitness cost to bacteria that harbor now a mobile genetic element and has to spend resources on its replication. Such collateral effect could affect directly the phenotype in study, thus explaining the apparent failure of phenotype rescues. We thus looked for another way to retrieve the WT allele to *ΔbcsC* strain without involving any mobile genetic element. We thus performed a transduction using P1 bacteriophage (Datsenko and Wanner, 2000). After PCR confirming the WT allele in its correct place in *E.coli* genome, we tested whether it resulted on a successful phenotype rescue or not. Surprisingly, we were still not able to find any contact-dependent growth advantage with the restored strain. Such result may be explained by some sort of independent mutation occurred during the construction of the ∆*bcsC* Keio strain that would be easily elucidated with the sequencing of the strain. To test this hypothesis we knocked out the *bcsC* gene in our *E.coli* Nal^R strain. Even without *bcsC* this strain displayed a growth advantage at high density competitions, confirming that the presumably association of *bcsC* and contact-dependent growth advantage mechanism was probably wrong. We leave these paradoxical results for future studies.

The predicted periplasmic zinc endopeptidase coded by *pqqL* has no known clarified specific function. However, a few gene expression studies state that *pqqL* is co-transcribed with *yddA* and *yddB* genes as a single mRNA (Subashchandrabose et al., 2013). YddA is an ABC transporter ATPase while YddB is an outer membrane β-barrel protein (not present in our list of predicted OMP) and the locus *yddAB* contain a putative Fur box suggesting that the cluster *yddABpqqL* can be involved in iron uptake (Engelen et al., 2011; McHugh et al., 2003; Subashchandrabose et al., 2013). In fact, iron uptake is an important step taking place in the lag phase of bacteria. According to (Rolfe et al., 2012) during lag phase of *S. enterica* a high number of genes involved in iron uptake are upregulated constituting, along with other metabolic regulatory machinery, an important step that prepares bacteria for exponential growth in a given environment. Since our work is based on early growth times (3.5 hours) it would not be surprising if the contact-dependent growth advantage was a mechanism acting by shortening the lag phase augmenting the iron uptake through *pqqL*-specific iron uptake machinery. This way, bacteria would be able to enter the exponential phase more rapidly before entering in nutrient starvation caused by high density competition. In fact (Petersen et

al., 2007) found the *pqqL* to be under positive selection in *E. coli* in a genome comparative study. Moreover, (Subashchandrabose et al., 2013) showed that *pqqL* is a fitness gene in an uropathogenic *E. coli* strain during systemic infection, being highly up regulated in an iron limited milieu that is human urine. However these authors concluded that *pqqL* expression was kept unaltered by iron levels alone (Petersen et al., 2007; Subashchandrabose et al., 2013). This role described for *pqqL* may be relevant given that iron promotes bacterial growth, hence being an important factor in infections by pathogenic bacteria (Guan et al., 2000; Paradkar et al., 2008).

 Unfortunately, it was not possible to restore the phenotype of the Δ*pqqL* Keio strain until the date of conclusion of this thesis, since we focused more on *bcsC* experiments.

The third candidate gene *yncD* to be tested for contact-dependent growth advantage, encodes a putative TonB-dependent receptor and thus it is recognized as a putative iron transport receptor (Zhai and Saier, 2002). Although being involved in iron metabolism, our results show that contact-dependent growth advantage is not affected by the deletion of this gene in *E. coli*.

It is worthy to notice the existence of two genes, *ydeS* and *yjiK*, with a significant higher fitness at high densities in tetrazolium-maltose medium experiments (see Table 3). The gene *ydeS* encodes a predicted fimbrial-like adhesin protein while *yjiK* has no known function. Our results suggest that the lack of YdeS gave mutant a growth advantage at high density. Possibly, the lack of this protein leads to an increase in contact between cells by decreasing the level of adhesion to surfaces.

In this work we describe and help resolving a contact-dependent mechanism that allows bacterial cells to sense other cells and growing faster if the bacterial density is high. To this mechanism we call contact-dependent growth advantage, as we show that it only occurs when cells are in contact with each other, excluding, for now, diffusible signal molecules. This seems to be a very good evolutionary strategy to overcome competitors. The fact that this signaling occurs by cell contact means that it occurs at the local level instead of being a signaling acting by distance. For a bacterial cell, it would be a bad strategy to signalize its presence to competitors at a larger scale, because it would alert the others for its presence. So, it is the cell that is willing to compete with others that has to have a mechanism to sense the others. This sensing mechanism has to be reliable and so direct cell-cell contact fulfills this condition.

According to our results, the growth promotion of bacteria seems to be associated with iron uptake, and the *ppqL* gene may be an important gene for the regulatory response of this mechanism. However, we still don't know what specific gene or genes are associated with signal transmission that allows bacteria to directly sense others. Further investigation will be necessary to unveil all the genetic route of this phenomenon which in turn would be a great

help in the clinical context since many infections with a little inoculum of the pathogenic bacteria would be aggravated if in presence of commensal neighboring populations which is the case of the human gut.

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