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David Ranava^{1*@\$}, Cassandra Backes^{1*#}, Ganesan Karthikeyan², Olivier Ouari², Audrey Soric³, Marianne Guiral¹, María Luz Cárdenas¹ and Marie Thérèse Giudici-Ortoni¹

Metabolic exchange and energetic coupling between nutritionally stressed bacterial species: the role of QS molecules

Running title: QS and metabolism control interactions in communities

¹CNRS, Aix-Marseille University, Bioenergetic and Protein Engineering Laboratory, Mediterranean Institute of Microbiology, IM2B, 31 Chemin Joseph Aiguier, 13009 Marseille, France.

²Aix-Marseille University, CNRS, UMR 7273, ICR, Marseille, France.

³Aix-Marseille University, CNRS; Centrale Marseille, M2P2, Marseille, France.

§To whom correspondence should be addressed: david.ranava@northwestern.edu

* DR and CB contributed equally to this work

@ Present address: Northwestern University, Feinberg School of Medicine, Department of Microbiology-Immunology 320 E. Superior Street Chicago, IL, 60611

Present address: MRC London Institute of Medical Sciences (LMS), Imperial College London, Hammersmith Hospital Campus, Clinical Research Building, 5th floor, Du Cane Road, London W12 0NN United Kingdom

Abstract

Formation of multi-species communities allows nearly every niche on earth to be colonized and exchange of molecular information among neighbouring bacteria in such communities is key for bacterial success. To clarify the principles controlling inter-species interactions, we previously developed a co-culture model with two anaerobic bacteria, *Clostridium acetobutylicum* (Gram-positive) and *Desulfovibrio vulgaris* Hildenborough (Gram-negative, sulfate-reducing). In conditions of nutritional stress for *D. vulgaris*, the existence of tight cell-cell interactions between the two bacteria induced emergent properties. Here we show that the direct exchange of carbon metabolites produced by *C. acetobutylicum* allows *D. vulgaris* to duplicate its DNA and to be energetically viable even without its substrates. We identify the molecular basis of the physical interactions, and how AI-2 molecules control the interactions and metabolite exchanges between *C. acetobutylicum* and *D. vulgaris* (or *Escherichia coli* and *D. vulgaris*). With nutrients *D. vulgaris* produces a small molecule that inhibits *in vitro* the AI-2 activity, and could act as an antagonist *in vivo*. Sensing of AI-2 by *D. vulgaris* could induce formation of an intercellular structure that allows directly or indirectly metabolic exchange and energetic coupling between the two bacteria

IMPORTANCE

Bacteria have usually been studied in single culture in rich media or in specific starvation conditions. However, in nature they coexist with other microorganisms, and build an advanced society. The molecular bases of the interactions controlling this society are poorly understood. Using synthetic consortium and reducing complexity allow us to shed light on the bacterial communication at the molecular level. This study presents evidence that QS molecule AI-2 allows physical and metabolic interactions in the synthetic consortium and provides new insights into the link between metabolism and bacterial communication.

Introduction

Microbial communities are ubiquitous and exert a large influence in geochemical cycles and health (1-4). In natural environments, stress factors such as nutrient deficiencies and the presence of toxic compounds can induce interactions between microorganisms from the same or different species and the establishment of

communities which can occupy ecological niches otherwise inaccessible to the isolated species (5, 6). Interactions between microorganisms can affect the behaviour of the community either positively or negatively (7).

For studying ecological communities, it is crucial to understand how the different members communicate with each other and how this communication is regulated. Interactions may occur either by release of molecules into the environment (8), or by direct contact between the microorganisms through structures like nanowires (9) or nanotubes (10). Dubey and Ben-Yehuda were the first to demonstrate a contact-dependent exchange of cytoplasmic molecules *via* nanotubes in *Bacillus subtilis* which contributes to proper colony (11, 12). The evolution of how metabolites came to be transferred between bacteria, and its functioning today, were both well described (13).

The type and extent of nutritional interactions between microbes partly determine the metabolism of an entire community in a given environment (14). Very little is known about the molecular basis of interactions between species, as this is difficult to investigate, especially in Nature, on account of community complexity. The use of a synthetic microbial ecosystem has considerable interest because the reduced complexity means that the investigation is more manageable, allowing not only identification of the specific community response, but also description of the different events at the molecular and cellular level (15).

To further investigate interactions between bacterial species we developed a synthetic microbial consortium constituted by two species: *C. acetobutylicum*, Gram-positive, and *D. vulgaris*, Gram-negative, sulfate reducing. There are both involved in anaerobic digestion of organic waste matter (16, 17). Glucose, a substrate that cannot be used by *D. vulgaris* (16), is the sole carbon source in this synthetic consortium. Under this condition, the consortium produces three times more H₂ than *C. acetobutylicum* alone, moreover *D. vulgaris* is able to grow even in the absence of sulfate, its final electron acceptor for the respiration process (18). Although *D. vulgaris* can ferment lactate, a metabolite produced by *C. acetobutylicum*, this process is greatly inhibited by high H₂ concentrations, preventing *D. vulgaris* from growing in the absence of methanogens (19). We observed a form of bacterial communication between adjacent cells of both types of bacteria by cell-cell interaction, in conditions of nutritional stress, with exchange in both directions of cell material, associated with the modification of the metabolism (18). In some cases, the

interactions between *C. acetobutylicum* and *D. vulgaris*, resembled those described by Dubey and Ben-Yehuda (10). Moreover, this type of cell-cell interactions has been seen also in other systems, which give support to its existence and functionality (10, 13).

Nutritional stress appears crucial to induce physical contact between bacteria, as this interaction was prevented by the presence of lactate and sulfate, nutrients of *D. vulgaris*. Furthermore, Pande et al (20) in a synthetic co-culture of *E. coli* and *Acinetobacter baylyi*, after depletion of aminoacids such as histidine and tryptophan by genetic manipulation, observed nanotubular structures between the auxotrophs allowing cytoplasmic exchange. As in our case, the communication between the mutants was prevented by the presence of the nutrients. The formation of nanotubes between aminoacid-starved bacteria might be a strategy to survive under aminoacid limiting conditions (13). Further evidence for the role of cell-cell connections to exchange nutrients can be found in these reviews (21-23).

Altogether these studies suggest that for some species, cell-cell interaction (either by tight cell junctions, nanotube formation, vesicle chains, or flagella) can allow them to overcome nutrient starvation and that many materials, from small molecules to proteins or plasmids, can be passed from one cell to another. However, this requires not only an energetic investment to establish the connecting structures, but also to find the suitable partners. Several questions arise: what sorts of signals are involved? What is the molecular mechanism? The fact that in several cases a nutritional stress induces interaction, but addition of nutrients prevents it, raises the question whether there is a distress signal that is released from the starving bacteria, and another (a quenching factor) when nutrients are there? Specific signalling between cells is of great importance in the proper development of the community and in its stability in the long term (24).

Here we partially answer these questions; in particular, we examine if the nutritional stress, which appears to be necessary, is also sufficient. We have investigated the possible role that quorum-sensing molecules could play in attaching the two bacterial cells involved in the consortia previously studied: *D. vulgaris* and *C. acetobutylicum* or *D. vulgaris* and *E. coli*, and we have examined how satisfactory the energetic state of *D. vulgaris* is in the co-culture when it is deprived of sulfate, and why the presence of nutrients prevents interaction between these bacteria.

Results

Tight bacterial interaction in the co-culture allows *D. vulgaris* to be metabolically active and to grow by using carbon metabolites produced by *C. acetobutylicum*

Based on metabolic and microscopic experiments, our previous results demonstrated that conditions of nutritional stress of *D. vulgaris* induce a tight interaction between *D. vulgaris* and *C. acetobutylicum*, in co-culture, which allows the exchange of cytoplasmic molecules and the growth of *D. vulgaris*. If the tight interaction is prevented when either *D. vulgaris* or *C. acetobutylicum* are confined in a dialysis tube, *D. vulgaris* cannot grow (18). A similar phenomenon was observed between *D. vulgaris* and *E. coli* DH10B (18) (Supplementary Fig. 1). This growth suggests an adequate energetic state of *D. vulgaris* in co-culture, despite of the lack of sulfate, its final electron acceptor, and shows that it can use metabolites from *C. acetobutylicum*, as *D. vulgaris* cannot use glucose (16).

To evaluate the physiological impact that *C. acetobutylicum* has on *D. vulgaris* in co-culture, we labelled *D. vulgaris* cells with Redox Sensor Green (RSG), a small molecule that can easily pass through the membranes of Gram-negative and Gram-positive bacteria, used as a respiration sensor to identify metabolically active cells. It has been tested on numerous bacteria, as an indicator of active respiration in pure or co-cultures (9, 25). If *D. vulgaris* became metabolically active due to its physical interaction with *C. acetobutylicum*, then a RSG fluorescence should be detected as for *D. vulgaris* cultivated in GY medium supplemented by lactate and sulfate (respiration) (Fig. 1a). As expected, in respiration conditions *i.e.* in the presence of lactate and sulfate, substrates of *D. vulgaris*, *D. vulgaris* in pure culture grows well and all the cells show intense RSG fluorescence, in contrast to the culture in GY medium, where there is no growth and very few cells fluoresce (Fig. 1a and b). As a control, we added CCCP, which by dissipating the proton gradient and abolishing the ATP synthesis, tightly impacts RSG fluorescence (Supplementary Fig. 2). When *D. vulgaris* is labelled with RSG, as above, and then cultivated for 20h with *C. acetobutylicum* in GY medium, despite the lack of sulfate it shows a significant RSG fluorescence indicating that the cells have enough reducing power to reduce redox green and are metabolically active (Fig. 1c-e). Furthermore, *C. acetobutylicum*, which

was not labelled by RSG at the beginning, also fluoresce intensely, indicating that RSG has been transferred from *D. vulgaris*.

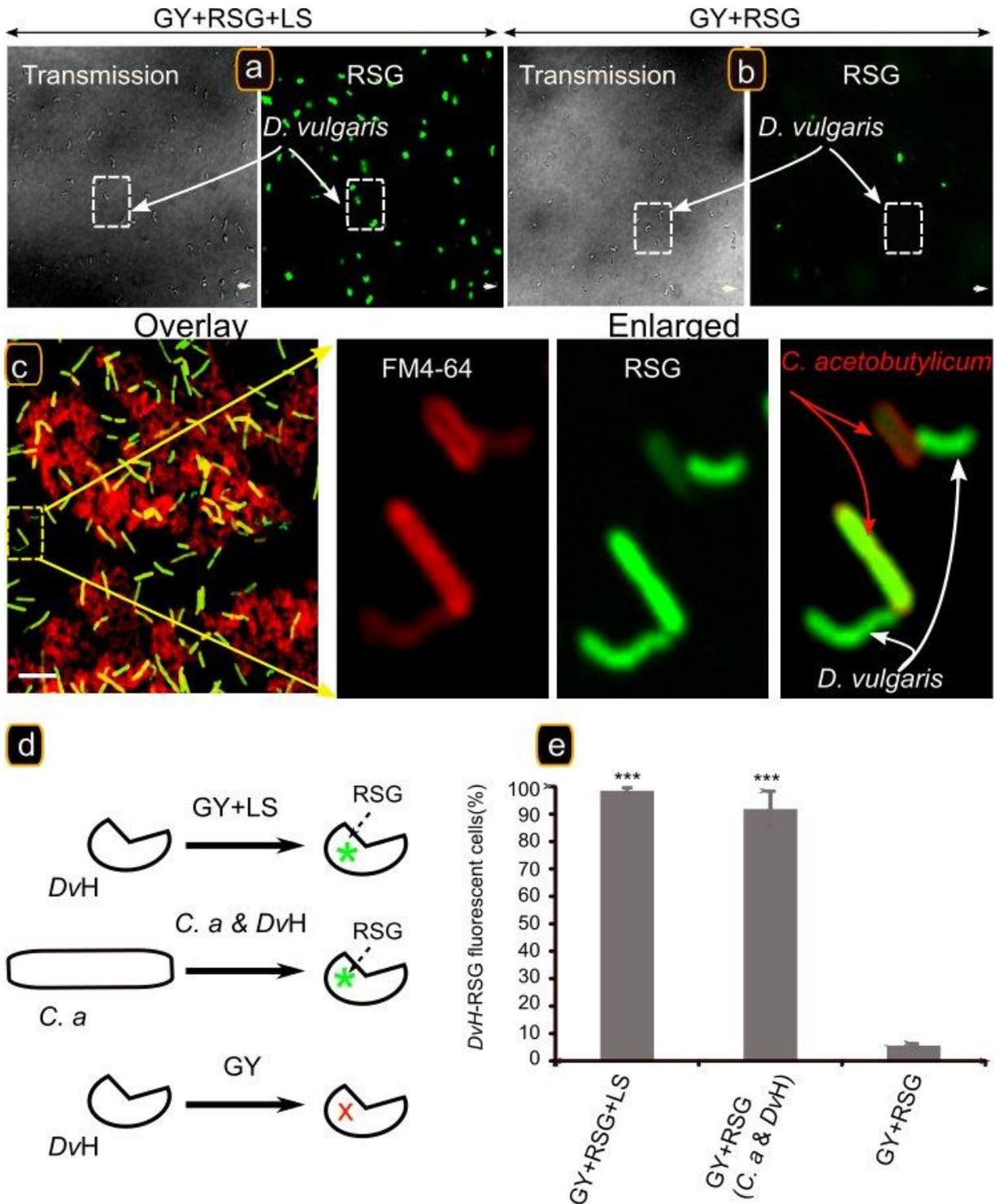


Figure 1: The presence of *C. acetobutylicum* is required for energetic activation of *D. vulgaris*. *D. vulgaris* growing exponentially in Starkey medium was washed twice and starved by incubation in GY medium, 20h at 37°C. A starved culture was divided into three subcultures and supplemented with 1µM of RSG (final concentration). The first was activated with 10mM of lactate/sulfate (LS) (a) and the second remained starved (b). The two subcultures were sampled and visualized by

fluorescence confocal microscopy after 20h incubation at 37°C). The third subculture was mixed with *C. acetobutylicum* cells. After 20h incubation at 37°C, the culture was sampled, left for 5min in contact with FM4-64 (in order to visualize the two strains) and visualized by fluorescence confocal microscopy (c). Scale bar, 2µm in all panels. Schematic representation of RSG activation or not in the cell in different conditions indicated (d). Percentages of *D. vulgaris* – RSG fluorescent cells in GY medium supplemented with lactate/sulfate, in GY medium with *C. acetobutylicum*, and in GY medium in pure culture (E) Data are represented as mean ± SD with n = 3, in comparison to *D. vulgaris* in pure culture in GY medium. p-values calculated in Tukey HSD tests, * p < 0.05; ** p < 0.01; *** p < 0.001. Abbreviations: *C. a.*, *Clostridium acetobutylicum*; *DvH*, *Desulfovibrio vulgaris* Hildenborough

To investigate carbon exchange between the two bacteria, we used Stable Isotope Probing, growing *C. acetobutylicum*, either alone or in co-culture with unlabelled *D. vulgaris*, on ¹³C-glucose medium. Total DNA were extracted from cells collected at the end of the exponential phase and ¹³C-DNA (heavier) and ¹²C-DNA (lighter) were separated by density-gradient centrifugation and examined on an agarose gel (Supplementary Fig. 3). Analysis of the different fractions using specific gene markers for the two bacteria shows that in the co-culture DNA from *D. vulgaris* is “heavy” (¹³C-labelled), indicating that metabolites derived from ¹³C-glucose were transferred between the two bacteria and used by *D. vulgaris*, despite the absence of sulphate. Quantitative PCR emphasized the presence of *D. vulgaris* ¹³C-labelled-DNA with *C. acetobutylicum* ¹³C- labelled-DNA in the same fraction (Fig. 2). A small amount of ¹²C- unlabelled-DNA (from the two bacteria) can be detected in this high-density fraction when an unlabelled co-culture is used, but not significant in relation to the total DNA and probably in line with the initial *D. vulgaris* inoculum. As *D. vulgaris* cannot grow on glucose or other hexoses, the ¹³C- labelled-DNA from *D. vulgaris* must have been formed using metabolites produced by *C. acetobutylicum*.

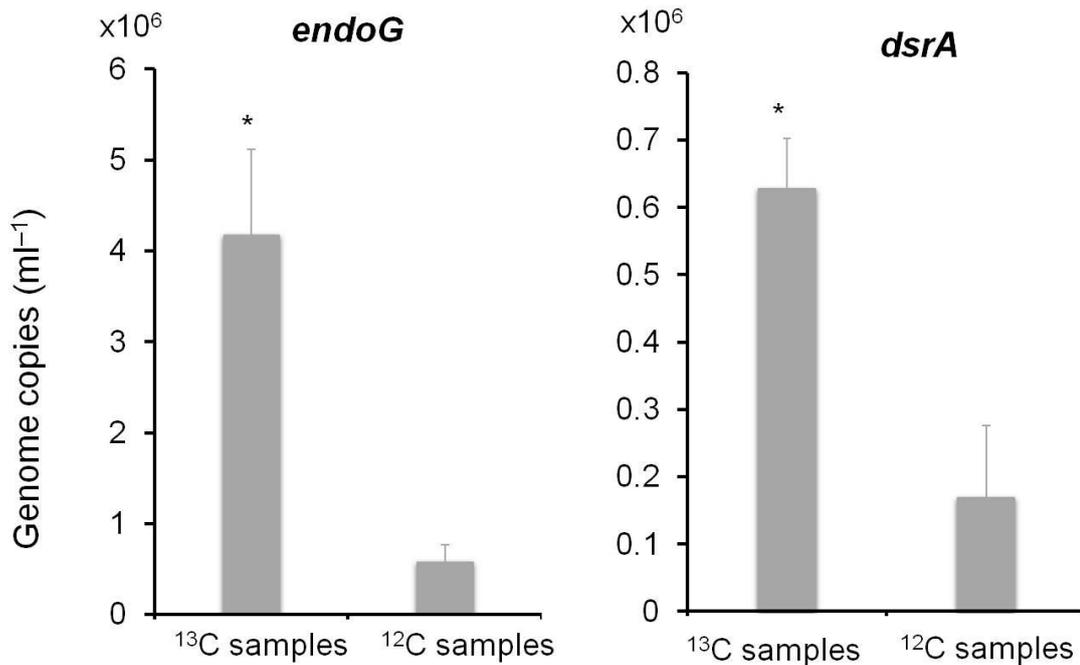


Figure 2: Quantification of *D. vulgaris* (*dsrA*) and *C. acetobutylicum* (*endoG*) on fractions (corresponding to the fractions 28 in Supplementary Fig. 3) containing ¹³C from *C. a* ¹³C + *DvH* ¹²C in ¹³C glucose medium DNA and on corresponding fractions containing ¹²C from *C. a* ¹²C + *DvH* ¹²C in ¹²C glucose medium DNA. Data are represented as mean ± SD with n = 3, in comparison to ¹²C samples. p-values calculated in Tukey HSD tests, * p < 0.05; ** p < 0.01; *** p < 0.001.

***C. acetobutylicum* produces AI-2**

As nutritional restrictions of *D. vulgaris* appear indispensable for inducing physical interactions between *C. acetobutylicum* and *D. vulgaris*, we investigated whether in addition to being necessary they were also sufficient, or if another element, such as quorum-sensing (QS) molecules, often associated with bacterial communication (26, 27) was required under our conditions. As the co-culture is composed by an association of Gram-positive and Gram-negative bacteria, AI-2, known to be involved in interspecies communication, appears as a good candidate to be tested (28). It is widely accepted as the universal cell-to-cell signal in prokaryotic microorganisms (29, 30). *Clostridium* species are known to develop QS systems based on peptides, but QS remains relatively unknown in sulfate-reducing bacteria (SRB) although inferences on the presence of putative QS systems in them can be made (31) and that more recently a role of QS (AHL molecules) in *D. vulgaris* biofilm formation, electron transfer and metabolism was proposed (32). However, no AI-2

signalling/sensing had been described in *C. acetobutylicum* or *D. vulgaris*. To determine whether *C. acetobutylicum* could generate AI-2-like activity, a cell-free supernatant of exponential phase *C. acetobutylicum* culture was tested for its ability to induce luminescence in *Vibrio harveyi* BB170 AI-2 reporter strain. This cell-free supernatant stimulated luminescence in a similar manner to cell-free supernatant of exponential phase *E. coli* DH10B (AI-2 producer) culture (Fig. 3a).

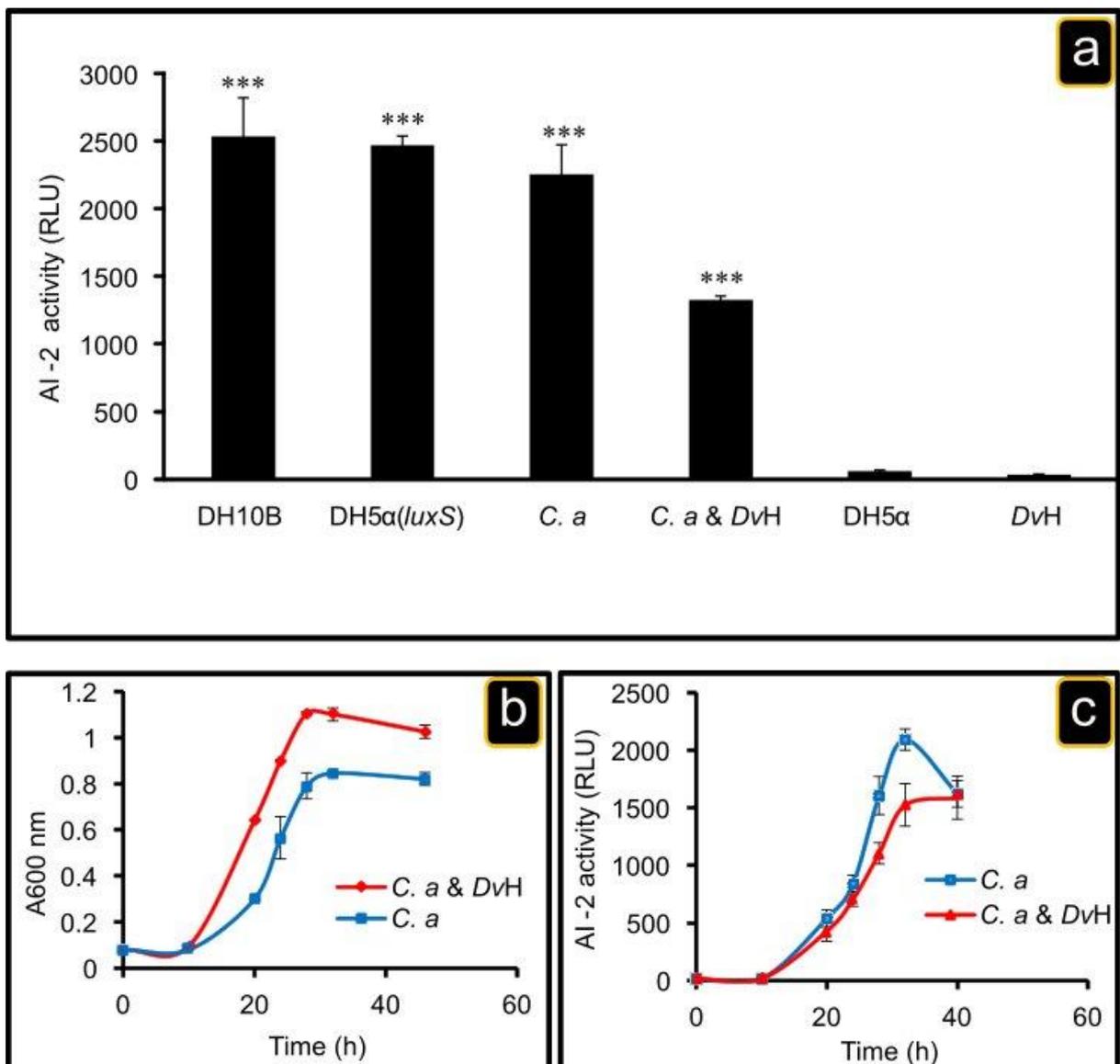


Figure 3: Extracellular AI-2 activity. (a) From left to right, pure culture of *E. coli* DH10B (AI-2 producer; positive control), pure culture of *E. coli* DH5α expressing *luxS* gene of *C. acetobutylicum*, pure culture of *C. acetobutylicum*, co-culture of *C. acetobutylicum* and *D. vulgaris*, pure culture of *E. coli* DH5α (AI-2 non-producer; negative control) all grown in GY medium to exponential phase (ie. OD₆₀₀ ≈ 0.8). At the right extreme *D. vulgaris* wild-type strain was grown in Starkey medium. Aliquots of different cultures were taken at exponential phase and filtered to remove cells. AI-2 activity in the cell culture supernatant was measured using the *V. harveyi* BB170

bioassay as described in Material and Methods. Data are represented as mean \pm SD (n = 3), in comparison to extracellular AI-2 activity from *D. vulgaris* wild type. p-values calculated in Tukey HSD tests, *p < 0.05; **p < 0.01; ***p < 0.001. (b, c): Time course of extracellular AI-2 accumulation in pure culture of *C. acetobutylicum* or in co-culture of *C. acetobutylicum* and *D. vulgaris*. Exponentially growing *C. acetobutylicum* in 2YTG medium and *D. vulgaris* in Starkey medium under anaerobic conditions were washed twice with fresh GY medium. Next, *C. acetobutylicum* was inoculated alone or mixed with *D. vulgaris* into GY medium at time zero and the aliquots were taken at indicated times. Cell growth was monitored by measuring the absorbance at 600nm (b), and AI-2 activity in cell-free culture fluids was measured in a pure culture of *C. acetobutylicum* or in co-culture, using the *V. harveyi* bioluminescence assay (c). AI-2 activity is reported as relative light unit (RLU) of BB170 bioluminescence. Abbreviations: *C. a*, *Clostridium acetobutylicum*; DvH, *Desulfovibrio vulgaris* Hildenborough ; DH5 α : *Escherichia coli* DH5 α ; DH5 α (*luxS*): *Escherichia coli* DH5 α expressing *luxS* gene ; DH10B : *Escherichia coli* DH10B.

Furthermore, AI-2 activity was also detected in the sterile filtered culture supernatant of exponential phase of *C. acetobutylicum* and *D. vulgaris* co-culture. The last step of the AI-2 biosynthetic pathway is catalysed by the *luxS* gene product (33), which is present in the *C. acetobutylicum* genome (CA_C2942) annotated as S-ribosylhomocysteinase and could encode for the LuxS protein. Genetic engineering on genus *Clostridium* remains difficult and requires the utilization of specific genetic tools. To avoid this, and to test if this gene is involved in AI-2 production, the putative *luxS* gene from *C. acetobutylicum* was introduced in *E. coli* DH5 α . which does not produce AI-2 due to a 60-amino-acid deletion stemming from a 1-bp deletion resulting in early truncation of *luxS* (formerly *ygaG*.) (34). *E. coli* DH5 α is commonly used as negative control for AI-2 production in different bacterial strains (34-36). As hypothesised, the cell-free supernatant of exponential phase of *E. coli* DH5 α (*luxS*) culture, expressing the *luxS* gene of *C. acetobutylicum* has AI-2 activity (Fig. 3a). In contrast, *D. vulgaris* does not have a homolog of the *luxS* gene and the cell-free culture supernatant collected from exponential phase of *D. vulgaris* culture, grown in Starkey medium, does not have AI-2 activity, this agrees with what is observed with the cell-free culture supernatant of *E. coli* DH5 α (Fig. 3a). As *D. vulgaris* does not produce AI-2, the AI-2 molecules present in the co-culture are likely to be produced by *C. acetobutylicum*. To verify whether *C. acetobutylicum* AI-2 production follows the growth, cell-free culture supernatant from *C. acetobutylicum* or from *C. acetobutylicum* and *D. vulgaris* co-culture taken at different times were used in the *V.*

harveyi bioluminescence assay, as described in Materials and Methods. As shown in Fig. 3b and c, *C. acetobutylicum* can synthesize functional AI-2 molecules, the synthesis following the growth in single culture as well as in co-culture, indicating that its production is independent of the presence of *D. vulgaris*.

Cytoplasmic exchanges of molecules between bacteria in the co-culture as well as metabolic activity of *D. vulgaris* depend on the presence of AI-2.

As *C. acetobutylicum* and *E. coli* DH10B, used in the previous studies (18), both produce AI-2, this raised the question of the situation if AI-2 was not present, that is if *E. coli* DH5 α , were used. So *E. coli* DH5 α or *E. coli* DH10B, both harbouring the pRSET-B mCherry plasmid containing the gene *mCherry*, was mixed with *D. vulgaris* cells lacking the *mCherry* gene but labelled with calcein or not, and the co-culture was analyzed by microscopy. Calcein-AM ester is a small non-fluorescent derivative of calcein which is sufficiently hydrophobic to pass readily through cell membranes. Once it is inside, the AM group is cleaved by esterases, yielding the more hydrophilic calcein (623Da), which is unable to cross membranes and is sequestered in the cytoplasm. The loss of the AM group also enables calcein to readily bind intracellular calcium, resulting in a strong yellowish-green fluorescence. When *D. vulgaris* cells were cultivated on GY medium with *E. coli* DH10B, more than 90% of *D. vulgaris* cells acquired a mCherry fluorescence signal after 20h of culture (Fig. 4a panel 2, Fig. 4b(2) and Supplementary Fig. 4 panel 1).

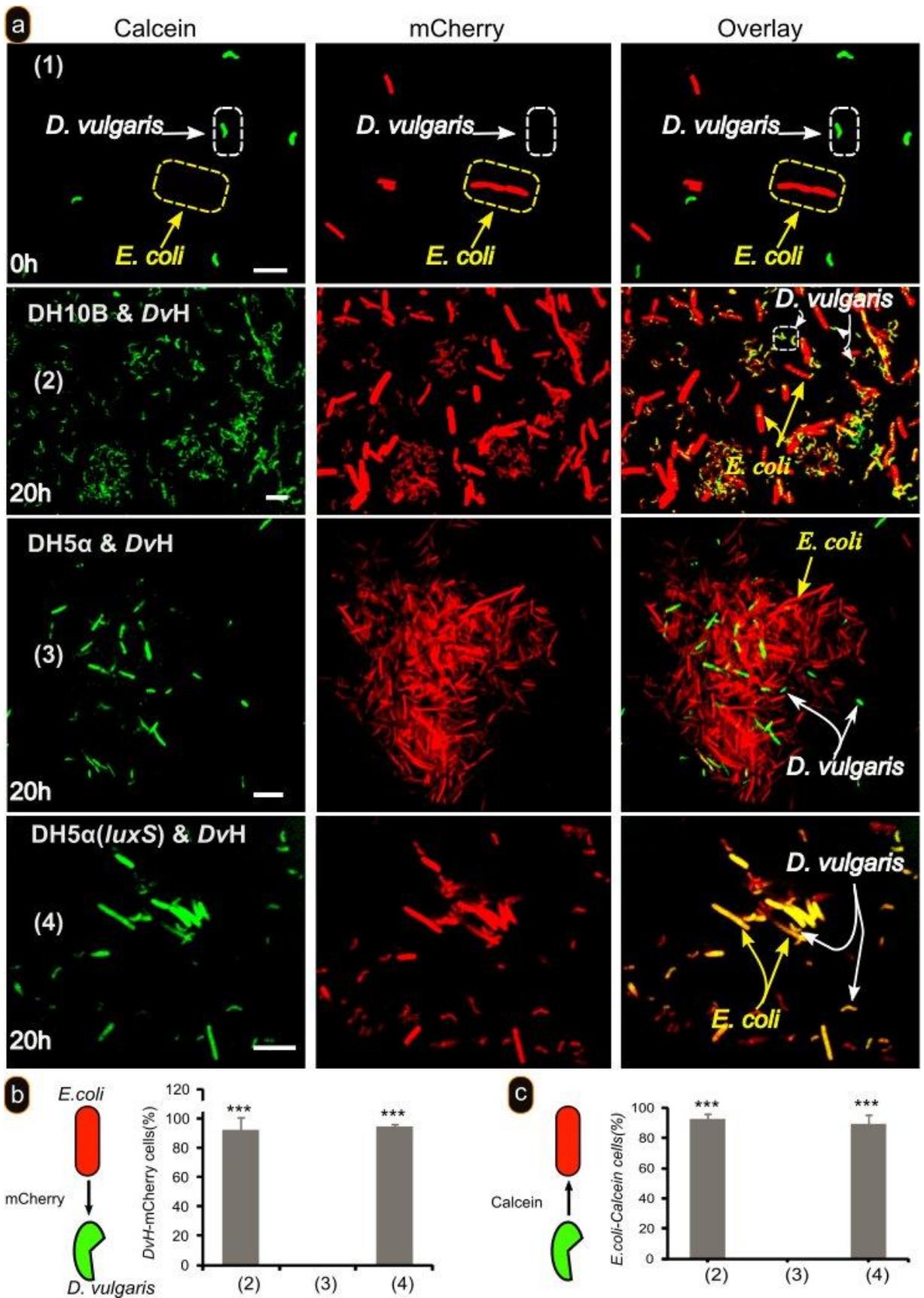


Figure 4: AI-2 is required for cytoplasmic molecules exchange between *D. vulgaris* and *E. coli*. *D. vulgaris* growing exponentially (OD of 0.6) in Starkey medium was labelled with calcein, washed with GY medium, and mixed with *E. coli*

strains DH10B (panel 2), DH5 α (panel 3) and DH5 α (*luxS*) (panel 4) labelled with mCherry and visualized by fluorescence confocal microscopy at time zero (panel 1) or after 20h incubation at 37°C (panels 2-4) in GY medium. Scale bar, 2 μ m in all panels (a). Percentage of *D. vulgaris* cells which acquired mCherry when co-cultured with different strains of *E. coli* (b). Percentage of *E. coli* cells which acquired Calcein when co-cultured with *D. vulgaris* (c). Data are represented as mean \pm SD with n = 3, in comparison to *E. coli* DH α (panel 3). p-values calculated in Tukey HSD tests *p < 0.05; **p < 0.01; ***p < 0.001. Abbreviations; *DvH*, *Desulfovibrio vulgaris* Hildenborough; DH5 α : *Escherichia coli* DH5 α ; DH5 α (*luxS*): *Escherichia coli* DH5 α expressing *luxS* gene; DH10B: *Escherichia coli* DH10B.

In contrast, no mCherry fluorescence was observed in *D. vulgaris* cells when they were cultivated with *E. coli* DH5 α (Fig. 4 panel 3, Fig. 4c (3) and Supplementary Fig. 4 panel 3). However, *D. vulgaris* cells (around 90%) became mCherry-fluorescent when they are co-cultured with *E. coli* DH5 α expressing *luxS* gene (Fig. 4 panel 4, Fig. 4b(3) and Supplementary Fig. 3 panel 2).

Taken together, these results suggest that AI-2 is essential for cell-to-cell communication and exchange of cytoplasmic molecules in co-culture, but not sufficient, as nutritional stress is also required. These results may explain why in the work reported by Pande et al (20) nanotubes, used to transfer amino acids, were observed between *E. coli* auxotrophe mutants, and between *E. coli* and *Acinetobacter baylyi* mutants, as in both cases there is the possibility of AI-2 produced by *E. coli*. In contrast, no nanotubes were observed between mutants of *A. baylyi* in which *luxS* is absent, in agreement with our genome bioinformatic analysis. So when AI-2 is not produced there may be no physical interaction even if there is a nutritional stress.

In view of the necessity of AI-2 to allow growth of *D. vulgaris* in the co-culture, we tested its effect on the energetic state of the cells with RSG, as in Fig. 1. Lack of AI-2 should prevent RSG fluorescence in *D. vulgaris* by preventing physical interaction between *E. coli* and *D. vulgaris* in GY medium. Cells of *D. vulgaris* were incubated with RSG as described above and mixed with *E. coli* DH5 α or with *E. coli* DH10B harbouring the gene of *mCherry* and the co-culture was analyzed by microscopy after 20h incubation at 37°C. *D. vulgaris* cells displayed a significant RSG fluorescence (90% of cells) when they are mixed with *E. coli* DH10B (Fig. 5 panel 1 and 5c(1)). In contrast, no RSG fluorescence was observed in *D. vulgaris* cells when co-cultured with *E. coli* DH5 α (Fig. 5 panel 2 and 5c(2)). Moreover *E. coli*

DH5 α does not show RSG fluorescence as *E. coli* DH10B and *C. acetobutylicum* (Fig. 1), which supports the absence of cytoplasmic exchange. In contrast, *D. vulgaris* cells co-cultivated with *E. coli* DH5 α complemented with *luxS* gene displayed RSG fluorescence (about 80% of cells) similar to that observed with *E. coli* DH10B (Fig. 5 panels 3 and 5c(3)). These results strongly support that the AI-2 molecule is important for physical interaction between *D. vulgaris* and *E. coli* and thus in metabolic activation of *D. vulgaris*. All these results suggest that *D. vulgaris* can detect AI-2.

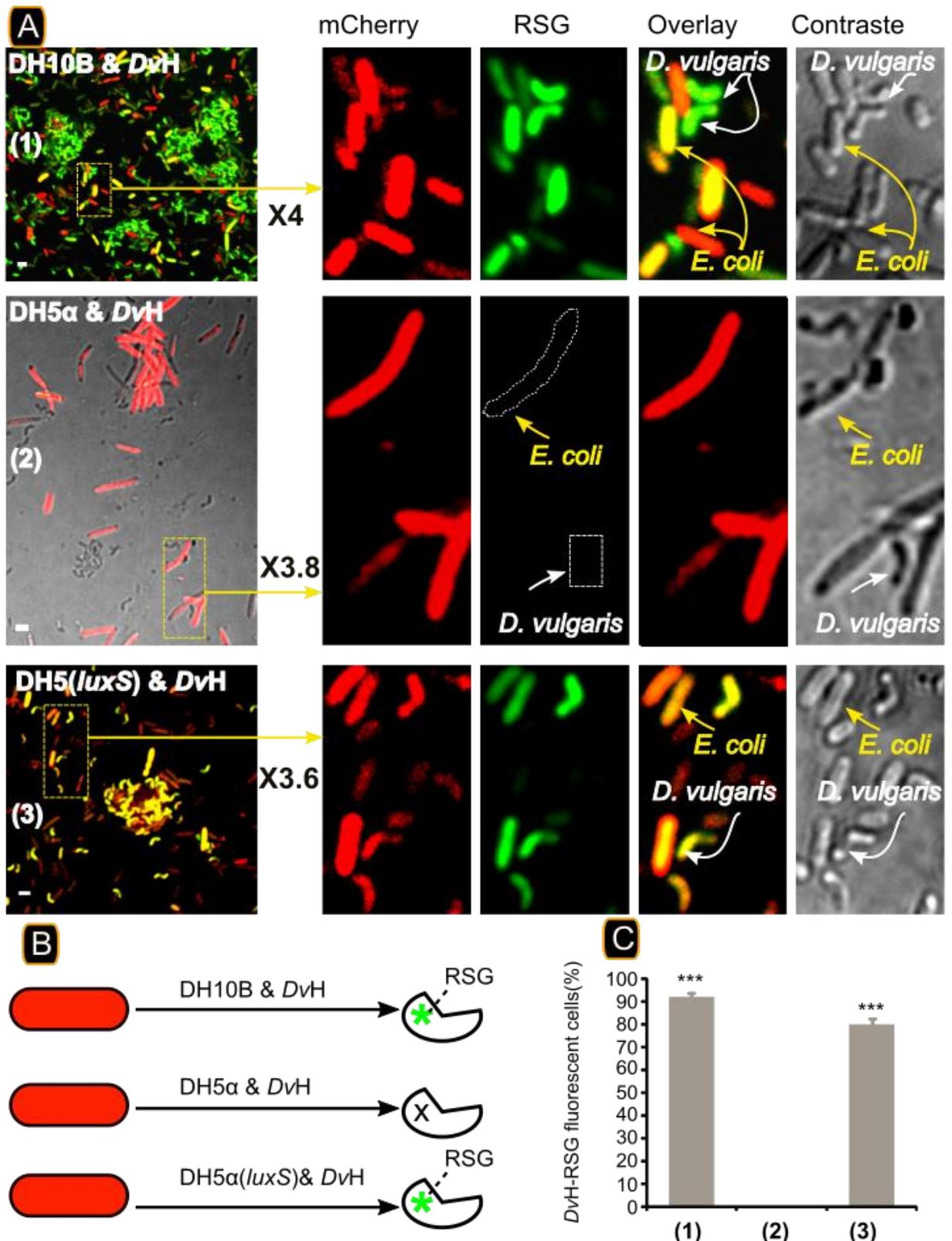


Figure 5: AI-2 is required for energetic activation of *D. vulgaris*. *D. vulgaris* was grown exponentially in Starkey medium, and was then washed twice and starved by incubation in GY medium, 20h at 37°C. A starved culture was divided into three subcultures and supplemented with 1µM of RSG (final concentration). The subcultures were incubated at 37°C for 1h and then *E. coli* strains DH10B (panel 1), DH5α (panel 2) and DH5α(*luxS*) (panel 3) labelled with mCherry were added. The

three subcultures were sampled and visualized by fluorescence confocal microscopy after 20h incubation at 37°C. Scale bar, 2µm in all panels (a). Schematic representation of RSG activation or not in the cell in different conditions indicated (b). Percentages of *D. vulgaris* – RSG fluorescent cells when co-cultured in GY medium with *E. coli* strains (c) Data are represented as mean ± SD with n = 3, in comparison to *D. vulgaris* co-cultured with *E. coli* DH5α in GY medium (non AI-2 producer). P-values calculated in Tukey HSD tests, * p < 0.05; ** p < 0.01; *** p < 0.001. Abbreviations: DvH, *Desulfovibrio vulgaris* Hildenborough; DH5α: *Escherichia coli* DH5α; DH5α(*luxS*): *Escherichia coli* DH5α expressing *luxS* gene; DH10B: *Escherichia coli* DH10B.

***D. vulgaris* produces an antagonist of AI-2 in the presence of sulfate and under respiratory conditions.**

The effect of AI-2 in the co-culture suggests that *D. vulgaris* can detect it. However, lactate and sulfate in the co-culture medium allow the growth of the two bacteria but prevent physical contact between the two bacteria and the transfer of cytoplasmic molecules (18) despite the fact that *C. acetobutylicum* and *E. coli* can produce AI-2. This suggests a regulatory mechanism linked to the presence of lactate and sulfate in the culture medium and/or to the sulfate respiration metabolism of *D. vulgaris*. At least two hypotheses may explain this: (i) in the presence of lactate and sulfate, *C. acetobutylicum* does not produce AI-2; (ii) *D. vulgaris* in the presence of sulfate produces one or several compound interfering with AI-2 activity.

The addition of lactate and sulfate to pure culture of *C. acetobutylicum* does not impair the production of AI-2 (Fig. 6a) and the *C. acetobutylicum* metabolism with 5--6 mM of butyrate produced. In contrast, in co-culture with *D. vulgaris*, the AI-2 activity detected in the sterile filtered culture supernatant of exponential phase, greatly decreased even in the presence of low quantity of lactate and sulfate (5mM), and was not detected by growing the co-culture in the presence of 10mM (Fig. 6a), suggesting that in sulfate respiratory conditions *D. vulgaris* could produce one or more metabolites that inhibit the activity of AI-2. To exclude the possibility that the supernatant taken from the culture grown in the presence of lactate/sulfate could inhibit the growth of the reporter strain, the growth of *V. harveyi* was measured. As shown in the supplementary figure 6, the addition of different supernatants does not affect the growth the *V. harveyi*

An important point is that in these conditions butyrate was produced (5mM), indicating that *C. acetobutylicum* is metabolically active and that the lack of AI-2 is not due to a metabolic inactivity of *C. acetobutylicum*.

To test the presence of molecules that could interfere with AI-2 activity in sulfate respiratory conditions, we followed the AI-2 activity present in the exponential phase supernatants culture of *E. coli* or *C. acetobutylicum* in the presence of increasing amounts of supernatant culture of *D. vulgaris* grown in Starkey medium for 30h. Sterile filtered culture supernatant of *D. vulgaris* inhibits AI-2 activity of *C. acetobutylicum* (Fig. 6b) and *E. coli* DH10B (Fig. 6c) cell-free supernatant, in a dose-dependent manner, indicating that *D. vulgaris*, in the presence of lactate and sulfate and independently of the presence of the other bacteria, released into the culture

medium an AI-2 inhibiting compound (or a mixture of such compounds)

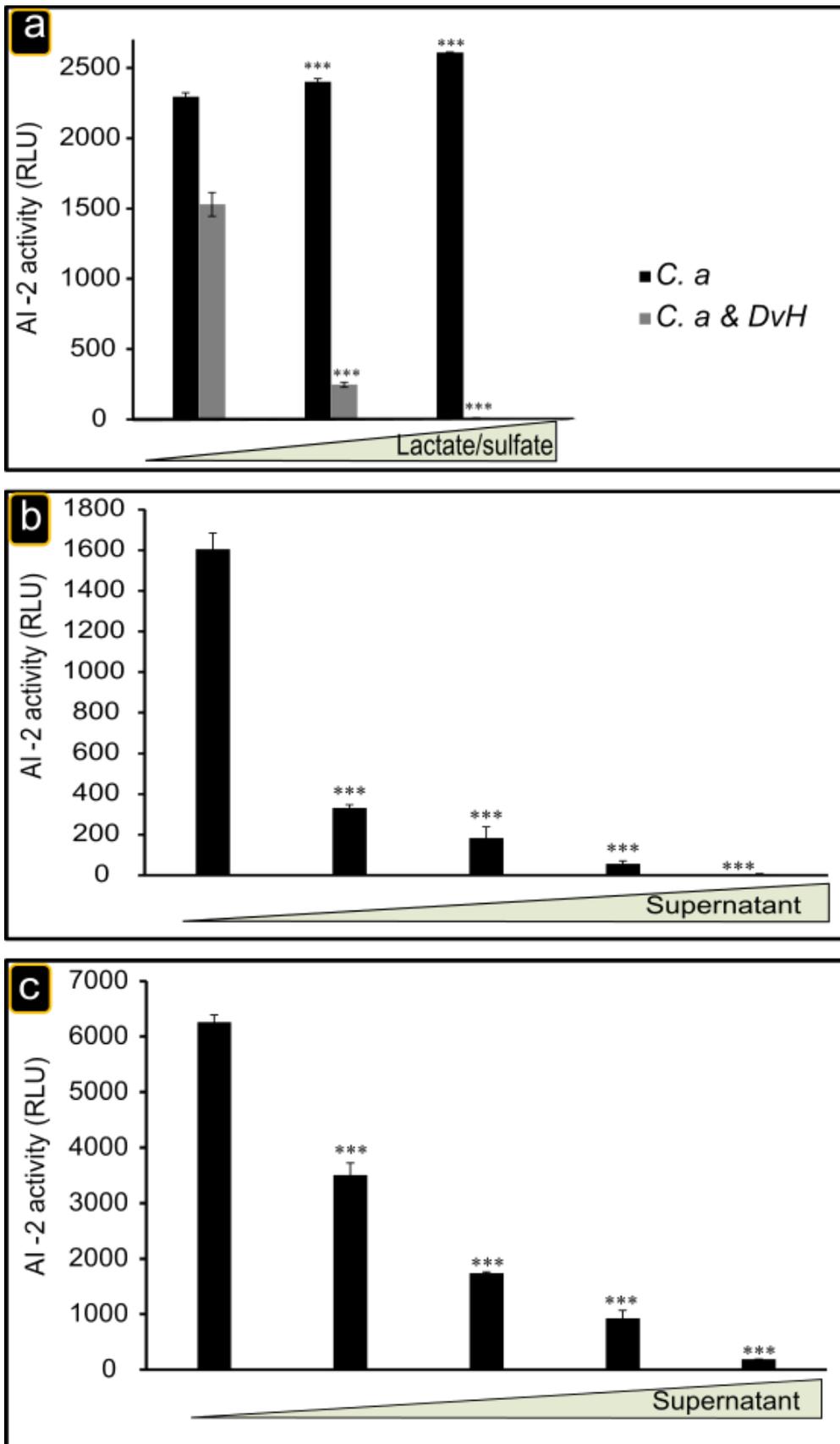


Figure 6: Inhibition of AI-2 activity. Addition of lactate and sulfate to the co-culture impaired AI-2 activity. Exponentially growing *C. acetobutylicum* in 2YTG medium or

D. vulgaris in Starkey medium under anaerobic conditions was washed two times with fresh GY medium. Next, *C. acetobutylicum* was inoculated alone (black) or mixed with *D. vulgaris* (gray) into GY medium supplemented with increasing of lactate/sulfate (5 and 10mM). After 30h of incubation at 37°C, the AI-2 activity was then analyzed using the *V. harveyi* reporter strain BB170 (a). *D. vulgaris* supernatant inhibits AI-2 activity. *C. acetobutylicum* (*C. a*) strain was grown in GY medium during 30h at 37°C. The activity of AI-2 in the filtered samples was then analyzed using *V. harveyi* reporter strain BB170 in the presence of various quantities (1, 2, 4 and 8µL) of *D. vulgaris* filtered (0.2µm) supernatant grown on Starkey medium for 30h (b). AI-2 activity is reported as relative light unit (RLU) of BB170 bioluminescence. *E. coli* DH10B strain was grown in GY medium during 30h at 37°C. The activity of AI-2 in the filtered samples was then analyzed using *V. harveyi* reporter strain BB170 in the presence of various quantities (1, 2, 4, and 8µL) of *D. vulgaris* filtered (0.2µm) supernatant grown on Starkey medium for 30h (c). Data are represented as mean \pm SD with n = 3, AI-2 activity measured for *C. acetobutylicum* in GY medium with 5 and 10mM of lactate/sulfate in comparison to *C. acetobutylicum* in GY medium (black) and *C. acetobutylicum* & *D. vulgaris* in GY medium with 5 and 10mM of lactate/sulfate in comparison to *C. acetobutylicum* & *D. vulgaris* in GY medium (gray) (a). In comparison to AI-2 activity measured for AI-2 produced by *C. acetobutylicum* and *E. coli* without *D. vulgaris* supernatant (b and c respectively). p-values calculated in Tukey HSD tests, * p < 0.05; ** p < 0.01; *** p < 0.001. Abbreviations: *C. a*, *Clostridium acetobutylicum*; *DvH*, *Desulfovibrio vulgaris* Hildenborough.

We followed the production kinetics of the AI-2 inhibiting compound by *D. vulgaris* by taking samples of sterile filtered supernatant at different times of the culture of *D. vulgaris* in Starkey medium. An AI-2 inhibitor is detected 3 hours after the beginning of growth (Supplementary Fig. 5). The production kinetics suggests a QS controlled expression. Microscopy analysis of *E. coli* and *D. vulgaris* co-culture in the presence of the *D. vulgaris* supernatant shows the loss of the interaction (Fig. 7). The sulfate respiration process is probably associated with the production of an antagonist or antagonists that inhibit the AI-2 activity. This production requires to the presence of sulfate, and is independent of the presence of *C. acetobutylicum* or *E.*

coli.

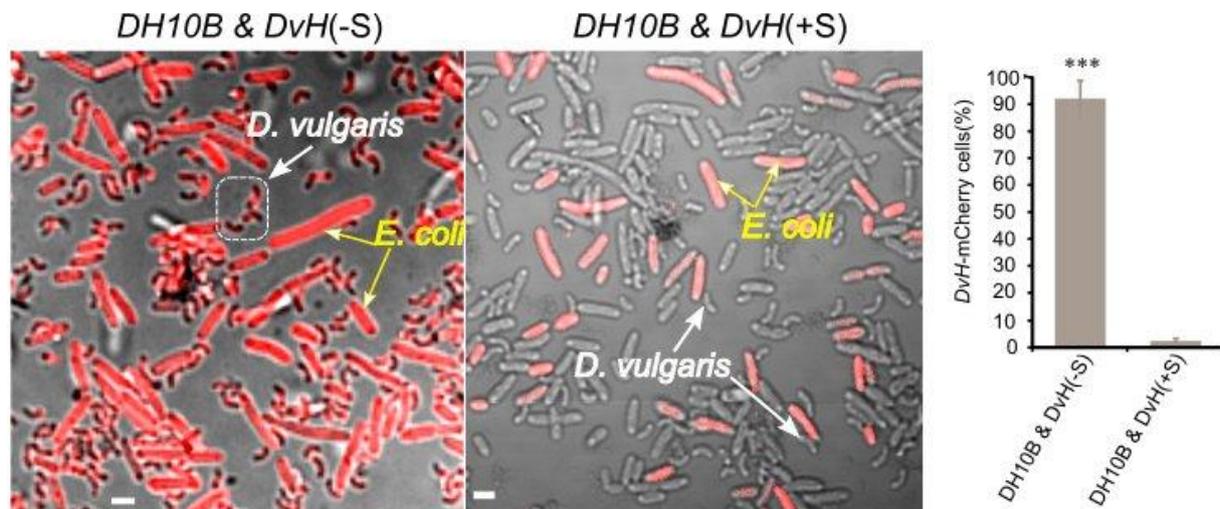


Figure 7: Impact of supernatant of *D. vulgaris* growing in Starkey on cytoplasmic molecule exchange. *D. vulgaris* growing exponentially in Starkey medium was washed with GY medium and mixed with *E. coli* DH10B (AI-2 producer) labelled with mCherry and grown in 5ml of GY medium supplemented (+S) or not(-S) with 100 μ l of *D. vulgaris* filtered (0.2 μ m) supernatant grown on Starkey medium. The culture was visualized by fluorescence confocal microscopy after 20h incubation at 37°C. Scale bar, 2 μ m in all panels. Data are represented as mean \pm SD with n = 3, in comparison to the percentage of mCherry *D. vulgaris* fluorescent cells when co-cultured with *E. coli* DH10B in GY medium supplemented with *D. vulgaris* Starkey supernatant. p-values calculated in Tukey HSD tests, * p < 0.05; ** p < 0.01; *** p < 0.001. Abbreviations: *C. a*, *Clostridium acetobutylicum*; *DvH*, *Desulfovibrio vulgaris* Hildenborough; DH10B: *Escherichia coli* DH10B.

To identify the compounds that interfere with the activity of AI-2, the cell-free supernatant of *D. vulgaris*, grown in Starkey medium for 30h, was analyzed by HPLC. All the major peaks (P1-P5) were recorded and their ability to inhibit AI-2 activity was determined on the supernatant of exponential phase *E. coli* DH10B culture. Under our test conditions, the peak P5 has a stronger effect on AI-2 activity than to P1-P4 (Fig. 8a and b). The peak (P5), which contains a 186Da molecule (Fig. 8a, indicated by black arrow) significantly, inhibits the AI-2 activity in a dose-dependent manner (Fig. 8c). Interestingly, we observed similar inhibition of AI-2 activity in the presence of peak 5 when using an *in vitro* synthesized AI-2. As the addition of *D. vulgaris* supernatant does not impair the growth of *V. harveyi* (Supplementary Fig. 6), the bioluminescence reporter strain, this result suggests the direct impact of molecules produced by *D. vulgaris* on AI-2 activity. The peak 5 analysis by mass spectrometry reveal a compound which has a molecular mass equivalent to that of AI-2 (192,9Da),

suggesting a similar type of molecule that could act as a competitive inhibitor. Taken together, these data show that the AI-2 receptor LuxP of *V. harveyi* can recognize the compound present in peak P5. However, it is still unclear whether this compound also binds to the AI-2 binding site or whether LuxP contains an independent binding site for this new compound. Different strategies for obtaining the structure did not succeed, probably because as with AI-2, there is an equilibrium between various forms (37).

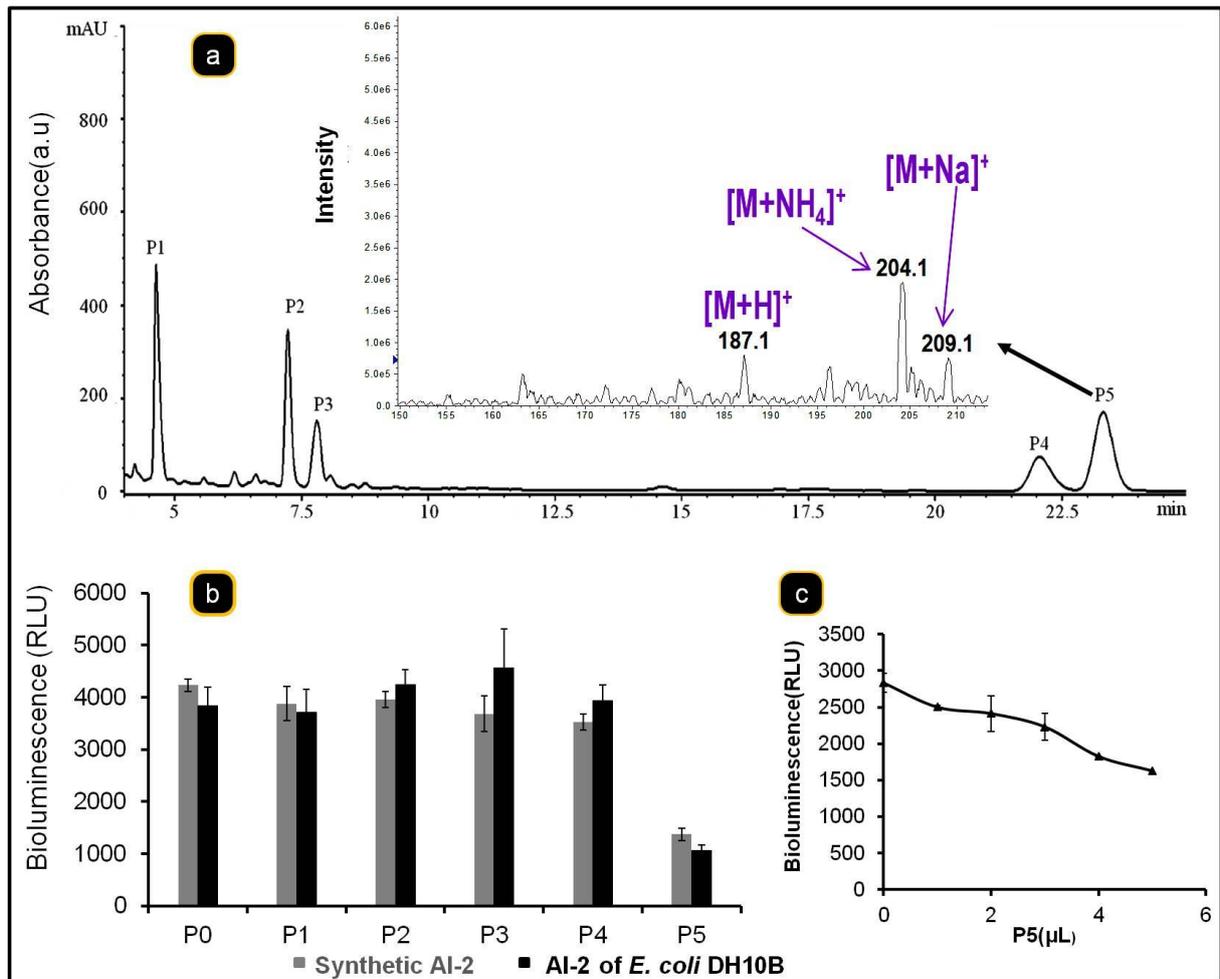


Figure 8: Identification of AI-2 inhibitor in *D. vulgaris* supernatant. *D. vulgaris* strain was grown in Starkey medium during 30h at 37°C and the filtered supernatant was analyzed by RP-HPLC (a). The peak P5 which presents an AI-2 inhibitor activity was analyzed mass spectrometry (A, indicated by black arrow). The AI-2 inhibitor activity of different peaks collected (P1 to P5) was determined on synthetic AI-2 ((S)-4,5-dihydroxy-2,3-pentandione, DPD) (2,5μM) or on AI-2 produced by *E. coli* strain DH10B grown on GY medium (b). To test the hypothesis that the P5 can compete with AI-2, *V. harveyi* reporter strain BB170 was grown in AB medium (100μl) supplemented with 500nM of synthetic AI-2 and in the presence of various quantities (0,1, 2, 4 and 5μL) of P5 and bioluminescence was measured after 4h (c).

Discussion

The bacterial community established in batch culture by *C. acetobutylicum* and *D. vulgaris*, or *E. coli* DH10B and *D. vulgaris*, and in conditions of nutritional stress for *D. vulgaris*: (i) exchanges metabolites, allowing a satisfactory energetic state and growth of *D. vulgaris*, and (ii) is regulated by AI-2 molecules that allow physical and metabolic interactions in the co-culture. Furthermore, in the presence of sulfate, *D. vulgaris* produces an AI-2 antagonist. Production of the antagonist is independent of the presence of *C. acetobutylicum* or *E. coli* and may prevent formation of other consortia. Altogether, our studies revealed how QS molecules coordinate interactions between species and this modulation follows the environmental stress. Moreover, they illustrate how experiments with multiple species or synthetic ecological models can provide new insight into bacterial sociability.

The presence of *C. acetobutylicum* or *E. coli* (DH10B) allows *D. vulgaris* to be energetically viable despite the lack of sulfate. The observation that without *C. acetobutylicum* or *E. coli* DH10B, *D. vulgaris* is not energized explains why the presence of one or the other of these two bacteria is indispensable for *D. vulgaris* growth. We previously demonstrated that proteins are able to be transferred via nanotube-like structures (18) which obviously could also be involved in the transfer of metabolites from one bacterium to another. However, we cannot exclude the possibility that these structures minimize the distance between the bacteria and create more or less a stable network that can prevent the diffusion of metabolites into the medium and thus increase their local concentration allowing the transfer by diffusion.

The presence of ^{13}C -DNA from *D. vulgaris* in conditions of co-culture with *C. acetobutylicum* using only ^{13}C -glucose indicates that *D. vulgaris* can grow on the metabolites produced by *C. acetobutylicum* and derived from ^{13}C -glucose if the two bacteria are in tight contact. Although *D. vulgaris* might just be using the metabolites produced by *C. acetobutylicum* and excreted to the culture medium, this is not supported by the observations that intercellular connections or at least in a very close environment appear indispensable to allow growth of *D. vulgaris* (18) as the presence of dialysis membrane that prevents the contact also prevents the growth. As in the co-culture, in the absence of sulfate (electron acceptor) and in conditions of tight contact between *D. vulgaris* and *C. acetobutylicum*, (i) *D. vulgaris* grows on the

metabolites produced by *C. acetobutylicum*, (ii) *D. vulgaris* cannot grow by fermenting lactate, because of inhibition by H₂; (iii) growth requires the existence of a respiratory metabolism. Thus *C. acetobutylicum* may be acting as final electron acceptor through a mechanism not yet elucidated.

How these kinds of interactions are initiated and controlled is at present poorly understood. Ben-Yehuda's group showed that YmdB is involved in the late adaptive responses of *B. subtilis* in the early stage of nanotube development (12). In various types of cells, it is the cell undergoing stress that develops the formation of nanotubes, suggesting that this might be directly induced by stress and constitutes a defense mechanism (38) as apparently also in this consortium.

Surprisingly, the role and the consequence of the QS molecules, well described in pure culture, are poorly investigated and understood in bacterial consortia, closer to those found in Nature. However, this has attracted the attention of researchers studying mixed cultures in bioreactors for treating waste water. Although the real mechanism involved in QS regulation, of complex microbial consortia, remained to be elucidated, studies of this type have shed light on it (39, 40). More recently one study investigated this question using a mathematical model to demonstrate how QS control the population trajectories in synthetic consortium (41).

The QS molecule AI-2 is crucial for metabolic interaction between the two bacteria of the co-culture, as there is no metabolic exchange in its absence. Thus *C. acetobutylicum* produces a molecule with AI-2 activity that had not been described before; furthermore, the *C. acetobutylicum* gene *luxS* can restore the AI-2 production by *E. coli* DH5 α . Our results explain why *E. coli* can connect to other bacterial cells to exchange cytoplasmic molecules.

Some organisms can produce and sense AI-2 whereas others only sense the AI-2 signal (42, 43). *D. vulgaris* appears to be in this last category, as it lacks the *luxS* gene, but appears to sense AI-2. This suggests the presence of AI-2 receptors in *D. vulgaris*. However, no genes similar to *lsrB* (coding for LsrB, protein receptor of AI-2 in enterobacteria) or to *luxP* (gene coding for LuxP, the protein receptor of AI-2 in *vibrionaceae*) are present in the genome of *D. vulgaris* (16). On the other hand, some bacteria can respond to an exogenous AI-2 signal (44, 45) despite lacking the genes *luxS*, *lsrB* or *luxP*. Furthermore, two proteins, AibA and AibB, can bind AI-2 in *Helicobacter pylori*. And the deletion of the genes (*hppA* and *modA*) encoding for these two proteins, induces deficiencies in chemotaxis and biofilm organization (46).

By bioinformatic analysis we have detected their homologues (*oppA* and *modA* respectively) in *D. vulgaris*, and experiments are in progress to study whether *D. vulgaris* internalizes AI-2, as preliminary results suggest. So, the issue of the cellular receptor of AI-2 remains an open problem and our results may contribute to clarify it.

Although the original QS concept was focused on the detection of cell density for the regulation of gene expression, studies in microbial ecology suggest a wider function. For example, the efficient-sensing concept (47) assumes that the ecologically relevant function of AI-2 sensing is to pre-assess the efficiency of producing extracellular effectors or “public goods”. Cooperative genes regulated by QS molecules can also be sensitive to nutrient conditions, suggesting that metabolic information is integrated into the decision to cooperate. The correlation between QS and cell activity rather than bacterial growth has been recently underlined in *D. vulgaris* (32). AI-2 molecules are involved in the mechanism stimulating viable but non-cultivable cell exits from dormancy, perhaps signalling to dormant cells when conditions are now favourable for growth (48, 49). This supports the idea that AI-2-dependent signalling reflects the metabolic state of the cell, and can function as a proxy for the production of effectors such as enzymes, or the formation of nanotubes. Integrating metabolic information with QS offers a possible mechanism to prevent cheating, as cells can only cooperate when they have the appropriate nutritional resources to do so, reducing the cost of cooperation to the individual cell (50).

We demonstrate the quenching of the AI-2 activity by a quorum quenching (QQ) molecule produced by *D. vulgaris* in the presence of lactate/sulfate. Quorum quenching has been suggested to be achieved in three ways: (i) blocking synthesis of autoinducers; (ii) interfering with signal receptors; and (iii) degrading the autoinducers (51-54). As we show competition between AI-2 present in *C. acetobutylicum* or *E. coli* supernatant, or even synthetic AI-2 and an AI-2 quencher, a small molecule presents in the *D. vulgaris* supernatant, we can exclude the first and the third hypotheses. Only a few AI-2 interfering mechanisms have been reported and most of them include synthetic molecules as quencher (37, 55, 56). It is proposed that C1 alkyl, analogues of AI-2, could compete with AI-2 for the LsrR transcriptional regulator in the *lsr* system (37, 57) and the presence of the competitor is linked to a decrease in AI-2 production. As AI-2 consists of a group of molecules in equilibrium, not a unique defined structure (37), analogy with enzymes and alternative substrates suggests that different types of AI-2 molecules may interact with a receptor, with only

some of them inducing a response. We cannot discard the possibility that the QQ molecule identified in *D. vulgaris* supernatant could bind to an AI-2 receptor in *C. acetobutylicum* and induce an effect at the level of gene transcription that could be translated into metabolic modification. Moreover, we also cannot discard the possibility that the QQ molecule identified represents a QS signal for *D. vulgaris*.

Our analysis provides new insights into metabolic prudence (58) and bacterial communication, and about how metabolic signals influence social behaviour but many details of its molecular implementation remain to be discovered. Which proteins detect the metabolic signals? How do they interact with QS regulation at the molecular level? However, one should also be cautious in using the word “signalling” because every change in a living organism affects every other, and thus acts as a signal of some kind (59, 60). In all of these studies it is important to keep in mind the ecological context, but the analysis of how the components of an ecological system influence one another has barely begun (61). Anyway, we can see in these microbial communities established, thanks to QS molecules, the preliminary steps in the evolutionary pathway of multicellular organisms and eukaryotes.

Materials and Methods

Media and growth conditions

Strains were grown to steady state in Hungate tubes under anaerobic conditions, in LB medium for *E. coli* DH10B and DH5 α , in Starkey medium (containing lactate and sulfate) for *D. vulgaris* (62) and 2YTG medium for *C. acetobutylicum* (63) to an optimal absorbance of 0.6. The growth medium (Glucose-Yeast extract (GY) medium) used for studying the consortium was prepared with glucose (14mM), 0.1% yeast extract, and supplemented with the similar inorganic nutrients used for the Starkey preparation (but with MgCl₂ instead of MgSO₄). GY medium was inoculated with either washed *D. vulgaris* or *C. acetobutylicum* or *E. coli* or with the combination of different strains to constitute an artificial consortium in a 1:1 ratio according to the absorbance at 600nm. *D. vulgaris* is not able to grow alone in GY medium (18). In some cases, the growth medium was supplemented with 5 or 10mM lactate or/and 5 or 10mM sulfate. The experiments were carried out at least in triplicate.

molecule, DH5 α (*luxS*). In some cases, unlabelled cells of *D. vulgaris* were mixed with *E. coli* labelled with mCherry. The mixture was diluted in 5ml fresh GY medium and put in a tube containing a coverslip and incubated at 37°C for 20h. The coverslip was removed after 20h of growth and bacterial cells attached to coverslip were visualized by fluorescence confocal microscopy as previously described by Benomar et al (18).

AI-2 activity assay

AI-2 activities of cell-free culture supernatants were measured by using *Vibrio harveyi* reporter strain BB170 as described by Bassler et al (65). Briefly, an overnight culture of *V. harveyi* (grown for 16h in AB medium) was diluted 1/5,000 in fresh AB medium (300mM NaCl, 50mM MgSO₄, 2% [wt/v] Casamino Acids, 10mM potassium phosphate [pH 7], 1mM L-arginine, 1% [wt/v] glycerol). The diluted cells (90 μ L) were added to 96-well plates (Corning) containing 10 μ L of the cell-free culture supernatant of *E. coli* or *D. vulgaris* or *C. acetobutylicum* obtained after centrifugation and filtration through 0.2 μ m membranes to remove bacterial cells, or synthetic AI-2 molecule. The microtiter plate was incubated at 30°C with shaking at 160rpm and bioluminescence was measured each hour over the course of 4-6h using a Tecan GENioS plate reader (Tecan, USA). AI-2 activity is reported as induction of bioluminescence which is expressed in Relative light units (RLU). The reported values represent the average bioluminescence stimulated by three independent preparations of cell-free culture fluids or synthetic AI-2 molecule. Similar experiments were performed in presence of various amounts of *D. vulgaris* culture supernatant in Starkey medium. Cell-free culture supernatants of *E. coli* or *D. vulgaris* or *C. acetobutylicum* were taken at exponential phase (OD₆₀₀ \approx 0.7/0.8).

Use of RedoxSensor Green as a probe for active respiration in *D. vulgaris*.

RedoxSensor Green (RSG) (Backlight™ RedoxSensor™ Green Vitality Kit, Life Technologies) was used to assess cellular respiration activity of *D. vulgaris*. *D. vulgaris* cells were taken from Starkey medium culture in mid-log phase, washed twice and starved by incubation in GY medium (without lactate and sulfate) for 20 hours at 37°C. Following starvation, *D. vulgaris* cells were harvested at room temperature by centrifugation at 4,000g for 10min, washed twice with GY medium

and diluted in 5ml fresh GY medium containing 1 μ M RSG reagent. The cultures were supplemented with 10 mM lactate and 10mM sulfate or not and bacterial cells were imaged by fluorescence microscopy following incubation at 37°C for 20h. After 20h of incubation at 37°C, the co-culture was left for 5min in contact with FM4-64 to visualize the bacterial membrane and to be able to see *C. acetobutylicum*, which was not labelled at the beginning of the experiment. Also, the effect of the electron transport chain uncoupler CCCP on RSG fluorescence was verified to further confirm the redox sensing functionality of RSG in *D. vulgaris* as previously reported for other bacteria (9).

Analysis and purification of the Antagonist AI-2 compounds from *D. vulgaris*

D. vulgaris cells were grown in Starkey medium under anaerobic conditions for 30h at 37°C. The culture was then centrifuged at 10,000rpm for 5min and filtered through 0.2 μ m membranes to remove the cells. The cell-free supernatants were stored at -20°C or immediately analysed by HPLC. The analysis was carried out on an Agilent 1200 HPLC system equipped with a UV detector and a refractometer (Agilent technologies). Separation (20-50 μ l of samples are injected) was achieved on an Agilent POROSHELL EC-C18 reverse-phase column (C18, 4.6x150mm, 2.7 μ m) set at 30°C. The compounds were eluted with 8% solution A (Acetonitrile + 1% (v/v) formic acid) and 92% of solution B (Deionized water + 1%(v/v) formic acid), at a flow rate of 0.6ml/min. Purified compounds were directly used for bioluminescence assay or lyophilized and stored at -20°C.

Mass spectrometry analysis

Analyzes of samples were performed with a 3200 QTRAP (Applied Biosystems SCIEX) mass spectrometer equipped with a pneumatically assisted atmospheric pressure ionization (API) source. The sample was ionized in positive electrospray mode under the following conditions: electrospray voltage (ISV): 5500 V; orifice voltage (OR): 10 V; nebulizing gas pressure (air): 10 psi. The sample was also ionized in negative electrospray mode under the following conditions: electrospray voltage (ISV): -4500 V; orifice voltage (OR): -10 V; nebulizing gas pressure (air): 10 psi. Mass spectra (MS) were obtained with a quadrupole analyzer.

Samples are dissolved in 300 μ L of acetonitrile and then diluted 1/10 in a 3 mM methanol solution of ammonium acetate. Sample solutions are introduced into the

ionization source by infusion (Harvard Apparatus syringe pump) at a flow rate of 10 μ L/min.

Carbon exchange by Stable isotope probing (SIP)

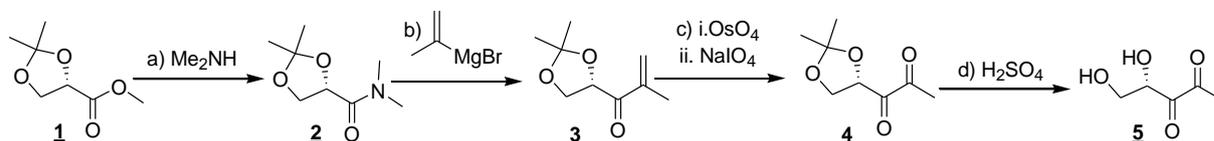
SIP method described by Neufeld et al (66) and derived from Meselson and Stahl was slightly modified. Glucose-yeast extract (GY) medium was prepared with D-glucose-¹³C6 (14mM) from Cortecnet and 0.1% yeast extract (¹²C), supplemented with similar inorganic nutrients as used for the Starkey preparation (MgCl₂ instead of MgSO₄) and N₂ in the headspace. ¹³C-GY medium was inoculated (10%) with either washed *C. acetobutylicum* enriched in ¹³C via 26 subculturing or *D. vulgaris* ¹²C at exponential growth phase, or with the two strains, to constitute an artificial consortium in a 1:1 ratio according to the absorbance at 600nm. At the end of the exponential phase, genomic DNA was extracted from the cell pellets using the NucleoBond AXG20 kit (Macherey-Nagel), and DNA purity and concentration were determined with the Nanodrop 2000c spectrophotometer (Thermoscientific).

To separate labelled/heavier (¹³C-DNA) from unlabelled/lighter (¹²C-DNA), density gradient centrifugation was performed in 5,1ml quick-seal tubes in a NVT 65.2 rotor (near vertical) using Optima L-90K centrifuge (Beckman coulter). CsCl medium having an average density of 1,72g/ml was loaded with 6 μ g of total extracted DNA. After centrifugation at 20°C for 66h at 41500rpm 169000g_{av}, each gradient (¹³C-labelled-*C. acetobutylicum* + ¹²C-unlabelled-*D. vulgaris* and ¹²C-unlabelled-*C. acetobutylicum* + ¹²C-unlabelled-*D. vulgaris*) was fractionated from the bottom to the top by displacement with mineral oil. DNA in each fraction was precipitated as described by Neufeld et al (66)

The presence and relative amount of DNA in fraction 28 (see supplementary Fig.3), from each bacterium were followed by qPCR. The primers used for the qPCR (*dsrA* and *endoG*) are listed in Supplementary Table 1. The reaction was performed with GoTaq mix and the PCR was carried out in a Techne Prime Elite thermal cycler as follows: 2min at 98°C for the initial activation of enzymes, 30 cycles of 30s at 98°C, 30s at 58°C and 2min at 72°C. Experiments were made in triplicate.

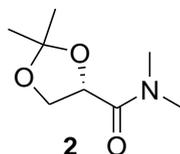
AI-2 synthesis

AI-2 was obtained in a four steps sequence starting from the commercially available methyl (S)-(-)-2,2-dimethyl-1,3-dioxolane-4-carboxylate adapting the reported procedures(67, 68).

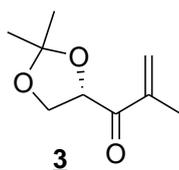


Scheme : a) Me_2NH , EtOH, 0°C - RT, 48hrs, 86% ; b) *i*-PropenylMgBr, Et_2O 48hrs, 46% ; c) i.OsO₄, NMO, CH_3COCH_3 : H_2O ; ii. NaIO₄, MeOH : H_2O , RT, 30min, 25% over 2 steps ; d) H_2SO_4 , D_2O : d_6 -DMSO, 0°C , 1hrs.

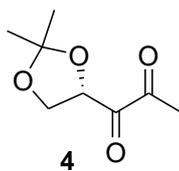
First, the methyl ester (**1**) was transformed into the amide (**2**) by reacting with dimethylamine in EtOH. The reaction of amide (**2**) with isopropenyl magnesium bromide gives olefin (**3**). Dihydroxylation of olefin (**3**) with catalytic osmium tetroxide in the presence of N-methylmorpholine-N-oxide and subsequent cleavage of generated diol with NaIO₄ produced ketone (**4**). Finally, the hydrolysis of dioxolane ring in acidic condition yields AI-2 (**5**) and its cyclic anomeric products. Spectral data were consistent with those previously reported. Details of each step are detailed below.



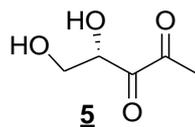
***N,N*-Dimethyl (S)- α,β -isopropylidene glyceramide (**2**)**. To the solution containing dimethylamine (20mL, 30 vol. % in ethanol) was added methyl (S) - α,β -isopropylidene glycerate (3g, 18.5mmol) at 0°C . The mixture was stirred for 24h. After adding an additional 10 mL of the dimethylamine solution, the stirring was continued for further 24 h. The volatile compounds were evaporated and the residue was purified by column chromatography to yield 2.8g of amide **2** (86%, colorless liquid); $R_f = 0.2$ (pentane / ethyl acetate, 3:2); $^1\text{H NMR}$ (400MHz, CDCl_3) $\delta = 4.62$ (t, $J = 6.65\text{Hz}$, 1H), 4.31 (dd, $J = 8.28, 6.53\text{Hz}$, 1H), 4.07 (dd, $J = 8.53, 6.78\text{Hz}$, 1H), 3.05 (s, 3H), 2.90 (s, 3H), 1.34 (s, 6H).



(S)-4-Methacryloyl-2,2-dimethyl-1,3-dioxolane (3): To a solution of amide **2** (1.74g, 10mmol) in anhydrous diethyl ether (10mL) at 0 °C under an Argon atmosphere, 21.0mL of a 0.5M solution of isopropenylmagnesium bromide in THF was added and stirred for 15minutes. The reaction mixture was quenched with 1M HCl and extracted 3 times with diethyl ether and the combined organic phase dried over Na₂SO₄ and evaporated to dryness. The residue was purified by chromatography on SiO₂ to afford **3** (0.8g, 46%). $R_f = 0.33$ (pentane / ethyl acetate, 9:1); ¹H NMR (300 MHz, CDCl₃) $\delta = 6.06$ (s, 1H), 5.91 (d, $J = 1.38$, 1H), 5.08 (dd, $J = 7.26, 6.03$, 1H), 4.22 (dd, $J = 8.34, 7.4$, 1H), 4.09 (dd, $J = 8.43, 5.94$, 1H), 1.89 (s, 3H), 1.41 (s, 6H).



1-(2,2-Dimethyl-[1,3]dioxolan-4-yl)propane-1,2-dione (4): The alkene (**3**) (40mg, 0.23mmol) was dissolved in a mixture of acetone/water (4mL/1mL). *N*-Methyl morpholine-*N*-oxide monohydrate (39mg, 0.23mmol) was added slowly and the reaction mixture was stirred at room temperature for 10min. Then 4% aqueous solution of osmium tetroxide (0.1mL, 0.011mmol) was added to the above reaction mixture and stirred overnight at room temperature. The mixture was quenched with Na₂SO₃ and extracted with dichloromethane. The organic layer was dried (Na₂SO₄), filtered and the filtrate concentrated under reduced pressure. The crude diol was used in next step as such. To the diol in methanol (3.5mL) and water (1.5mL) was added sodium periodate (0.146g, 0.69mmol) and stirred at RT for 30min. The reaction mixture was diluted with water and extracted with dichloromethane. The organic layer was dried (Na₂SO₄), filtered and the filtrate was concentrated under reduced pressure. The residue was purified by chromatography on SiO₂ to afford **4** (15.9mg, 40% over 2 steps). $R_f = 0.65$ (pentane / ethyl acetate, 6:4); ¹H NMR (400 MHz, CDCl₃) $\delta = 5.14$ (dd, $J = 7.8, 5.8$, 1H), 4.37 (t, $J = 8.4$, 1H), 4.0 (dd, $J = 9.00, 5.4$, 1H), 2.39 (s, 3H), 1.47 (s, 3H), 1.42 (s, 3H).



(S)-4, 5-Dihydroxy-pentane-2, 3-Dione (**5**): To a solution of diketone **4** (3.0mg, 0.017mmol) in D₂O (0.5mL) and d₆- DMSO (0.2mL) under an Argon atmosphere, H₂SO₄ (0.005mL) was added and stirred at RT for 1h. The reaction mixture was quenched with 5mg Na₂CO₃, filtered to get 0.0249M solution of Al-2 (**5**) and its cyclic anomeric products in D₂O and d₆- DMSO. Spectral data were consistent with those previously reported. ¹H-NMR (300MHz, D₂O): 4.25 (t, *J*= 6.4Hz, 1H), 4.10-4.02 (m, 2H), 3.93 (dd, *J*= 3.4, 5.6Hz, 1H), 3.76-3.67 (m, 2H), 3.84-3.81 (m, 1H), 3.53 (dd, *J*= 7.5, 11.6Hz, 1H), 3.45 (dd, *J*=5.4, 9.2Hz, 1H), 2.27 (s, 3H), 1.34 (s, 3H), 1.31 (s, 3H). HRMS (ESI-TOF) *m/z*: [M - H]⁻ calcd for C₅H₇O₄⁻ 131.0350; found 131.0354.

Imaging and quantification of microscopy images

Cells attached to the coverslip were observed by the Confocal Olympus FV1000 microscope (Japan) using the UPLSAPO 100X objective (oil immersion). The excitation and emission wavelengths are 488 nm and 515 nm for calcein and RSG, and 558 nm and 583 nm for mCherry. The laser beams were activated in sequential mode to avoid fluorescence overlaps. The microscopy fields analyzed were obtained from 3 biological replicates obtained independently containing between 100-150 cells. These fields were chosen according to the local cell density. Since we made a morphological discrimination to identify the different bacterial species, we scanned the entire coverslip to find microscopy fields with small clumps of bacteria where the morphology of each was observable. They were counted manually using ImageJ software.

Statistical analysis

All the data were obtained from at least 3 biologically independent replicates. Data analysis was performed using the R statistical analysis software v3.6.0. Statistical data were accomplished using R base function “aov” for Tukey test. Tukey test was used because it is a statistical test to perform a multiple comparison in one step. Data were compared with base condition and significances were estimated with *p*-values : * *p* < 0.05; ** *p* < 0.01; *** *p* < 0.001. In all cases, *p* values < 0.05 was

considered significant. For details regarding statistical tests, see supplementary table 2.

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Supplementary Table 1: Primers used for PCR quantification for *D. vulgaris* and *C. acetobutylicum*

Strains	Genes	Sequences 5'- 3'
<i>D. vulgaris</i>	<i>dsrA-fwd</i> <i>dsrA-rev</i>	GAATTCGCCTGCTACGACTC TCCTTCCAGGTACCGATGAC
<i>C. acetobutylicum</i>	<i>endoG-fwd</i> <i>endoG-rev</i>	ATGCGGCTACAGCTGAACT ATTCTTTGCACCGGTGTCTC

Supplementary table 2

Fig. 1e	<i>D.vulgaris</i> fluorescer	sqr		
Cab+DvH-RSG(FM4-64)	92.072306	3.729872576		
DvH -RSG+Lactate/sulfate	98.62396038	0.701246665		
DvH -RSG without Lactate/sulfate	5.778061386	0.423639888		
Fig. 2	fractions	genome copies	sqr	
dsrA	f13C	627265.6679	74793.20234	
dsrA	f12C	169173.4765	107211.4977	
endoG	f13C	4.18E+06	9.50E+05	
endoG	f12C	5.77E+05	1.93E+05	
Fig. 3a	moy AI-2 activity	sqr		
DH10B	2535	167.003992		
DH5+LuxS	2491	40.61198509		
C. a	2255	127.4414898		
C. aDvH	1325.333333	18.55921454		
DH5a	61	5.033222957		
DvH	34.33333333	3.179797338		
Fig . 4	<i>D.vulgaris</i> fluorescer	sqr	<i>E .coli</i> fluorescents	sqr
DH10B +DvH	92.40671576	4.577081152	92.71624802	1.97201277
DH5aLuxS+DvH	94.69515689	0.926391377	89.21247563	3.39795829
dh5a+DvH	0	0	0	0
Fig. 5c	<i>D.vulgaris</i> fluorescents			
DH10B+DvH	92.02128464	0.86568809		
DH5a+DvH	0	0		
DH5aLuxS+DvH	79.73385264	1.551688763		
Fig. 6a	Lactate/sulfate	moy AI-2 activity	sqr	
C. a	0mM	2295	17.21433511	
C. a	5mM	2403	12.7410099	
C. a	10mM	2609.666667	2.962731472	
C. a + DvH	0mM	1530.666667	49.00793587	
C. a + DvH	5mM	248	10.39230485	
C. a + DvH	10mM	10	0.577350269	

Fig. 6b	DvH supernatant	moy AI-2 activity	sqr	
C. a	0ul	1604.333333	46.1747887	
C. a	1ul	330.3333333	6.437735972	
C. a	2ul	182.6666667	32.27141838	
C. a	4ul	54.66666667	6.227180564	
C. a	8ul	7	1	
Fig. 6c	DvH supernatant	AI-2 activity	sqr	
E. coli DH10B	0ul	6263	74.80864478	
E. coli DH10B	1ul	3503	130.001282	
E. coli DH10B	2ul	1563.666667	176.606279	
E. coli DH10B	4ul	843.6666667	102.1377066	
E. coli DH10B	8ul	160.3333333	28.81164888	
Fig. S4	<i>D. vulgaris</i> fluorescer	sqr		
DH10B+DvH	94.71268076	2.144019364		
DH5aLuxS+DvH	93.28478302	1.881735585		
DH5a+DvH	0	0		
	C. a + supernatant	sqr	DH10B + supernatant	sqr
C	2114.333333	101.6666667	3419	54
0h	2206.666667	98.41634914	3335.666667	46.4626493
3h	104.6666667	64.34369519	1487.333333	93.0238918
6h	52.33333333	13.86041526	1459	107.704844
9h	50.33333333	9.527737285	1370.5	1.5
12h	4.666666667	2.905932629	930	274.049266
26h	1	1	3.5	2.5
30h	7	4	0	0
Fig. S6	Lactate/sulfate	<i>V. harveyi</i> growth	sqr	
<i>V. harveyi</i> with C. a supernatant	0mM	0.0373	0.001014889	
<i>V. harveyi</i> with C. a supernatant	5mM	0.0372	0.001509967	
<i>V. harveyi</i> with C. a supernatant	10mM	0.037366667	0.000945163	
<i>V. harveyi</i> with C. a/DvH supernatant	0mM	0.001014889	0.005071489	
<i>V. harveyi</i> with C. a/DvH supernatant	5mM	0.001509967	0.003879863	
<i>V. harveyi</i> with C. a/DvH supernatant	10mM	0.000945163	0.002650157	
<i>V. harveyi</i> no supernatant	/	0.038633333	0.00322542	