Microniches in biofilm depth are hot-spots for antibiotic resistance acquisition in response to *in situ* stress

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Abstract

Class 1 integrons play a major role in antibiotic resistance dissemination among Gram-negative bacteria. They are genetic platforms able to capture, exchange and express antibiotic resistance gene cassettes. The integron integrase, whose expression is regulated by the bacterial SOS response, is the key element of the integron catalyzing insertion/excision/shuffling of gene cassettes. We previously demonstrated that the basal level of integrase expression and in consequence, its activity, is increased *via* the starvation-induced stringent response in the biofilm population. However, biofilms are heterogeneous environments where bacteria are under various physiological states. Here we thus analyzed at the bacterial level, the SOS response and integrase expression within the biofilm, using confocal microscopy and flow cytometry. We showed that in the absence of exogenous stress, only a small number of bacteria (~ 1%) located in the depth of the biofilm induce the SOS-response leading to a high level of integrase expression, through both a stringent response-dependent and -independent manner. Our results thus indicate that few bacteria located in microniches of the biofilm depth undergo sufficient endogenous stress to promote the acquisition of antibiotic resistance, forming a reservoir of bacteria ready to rapidly resist antibiotic treatments.

Introduction

In natural settings, bacteria most often live in complex communities attached to surfaces known as biofilms [1]. Biofilms are highly heterogeneous environments where bacteria experience local gradients of physical (temperature, pH) and biological (nutrients, oxygen, waste products) parameters, resulting in microniches of distinct bacterial subpopulations [2]. Early biofilm characterization studies used the biofilm as a whole, which provided global information averaging the entire biofilm bacterial population [2,3]. In the last decades, the use of fluorescent reporter genes in combination with confocal scanning laser microscopy (CLSM) has proven to be particularly useful for monitoring gene expression heterogeneity within biofilms [4–8]. Fluorescence-activated cell sorting (FACS) offered complementary information allowing to separate biofilm subpopulations to further characterize them independently [9–11]. All these techniques have helped to show that biofilms favor horizontal gene transfer [12], the main driving force for the emergence and dissemination of antibiotic resistance. One of the most striking characteristics of the biofilm is its recalcitrance towards antibiotics and host defenses, leading to treatment failure and infection recurrence[13].

Resistance integrons (RI) are low-cost structures allowing bacteria to rapidly adapt to antibiotic pressure [14,15]. They play a major role in the spread of antibiotic resistance among Gram-negative bacteria [14]. Integrons are genetic platforms able to capture, exchange and express resistance genes embedded within gene cassettes (GC) from a common promoter Pc [16]. The integrase, *IntI*, is the key element of the integron system, catalyzing GC insertion and excision through site-specific recombination [17]. Among the five classes of RIs identified based on the *IntI* amino acid sequence, class 1 RIs are the most frequently reported in antibiotic-resistant clinical isolates [18,19]. The expression of the class 1 integrase, *IntI1*, is controlled by the bacterial SOS response, a global regulatory network addressing DNA

damages and mainly governed by the transcriptional repressor LexA [20,21]. We recently reported that in the absence of exogenous stress, the expression level of the *intI1* integron integrase, as well as that of the SOS regulon gene *sfiA*, was higher in a continuous biofilm culture than in a planktonic culture [22]. This increase depended on the RelA and SpoT proteins, the regulators of the stringent response (stress response induced upon starvation and whose effectors are the alarmones (p)ppGpp [23]). However these data were obtained from the whole biofilm and may not reflect what actually happens within its heterogeneous structure, where not all bacteria are subjected to nutrient starvation [24]. Herein, using CLSM and FACS approaches, we could refine our previous observation, and showed that actually only a very small number of bacteria located in the depth of the biofilm expressed *intI1* and *sfiA* genes at a high level in both a *relA/spoT*-dependent and -independent manner in the absence of exogenous stress. Most importantly our results highlight that endogenous stress in specific microniches in the depth of the biofilm would be sufficient to form a reservoir of bacteria fully ready to develop antibiotic resistance upon antibiotic treatments.

Materials and methods

Strains and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 1. Strains were grown under planktonic or biofilm conditions at 37°C in Luria-Bertani (LB) medium supplemented with kanamycin (Km; 25µg/ml) when required.

Biofilms in continuous culture were grown for 24 h in a glass microfermentor containing a removable spatula, as previously described [22]. Briefly, the microfermentor were inoculated by dipping the spatula for 2 min into 15 ml of over-night bacterial culture containing 1X10⁹ cells/ml, followed by a brief rinse in LB medium before insertion in the microfermentor and

start of the culture. After 24h, the biofilm was resuspended by vigorously shaking the microfermentor, and the biofilm biomass was estimated by determining the optical density at 600 nm (OD₆₀₀).

For planktonic culture, 100 µl of the culture used to inoculate the microfermentor was diluted in 10 ml of LB and grown for 24 h at 37°C with shaking.

Construction of the MG1656 λ att::cherry F' strain

The *E. coli* MG1656 λ att::mcherry strain, expressing red fluorescent protein (mCherry), was constructed by integrating a *km-frt-mcherry* cassette at the *E. coli* λ att site in a three-step-PCR-based approach, and removing the Km resistance gene with the FLP recombinase as previously described [25]. Primers used to construct and verify the strain, are listed in Table S1. The F' plasmid was introduced by conjugation in this strain from the NEB5-alphaF' strain.

Plasmid construction

pPsfIAgfp⁺ and pPsfIA**gfp*⁺ were constructed by amplifying the *gfp*⁺ gene (encoding a GFP variant with increased fluorescence intensity[8]) from plasmid pGZA1 (Table 1) using primers PsfiA-gfp(+)-INF-5 and PsfiA-gfp(+)-INF-3 and cloning the amplified fragments into plasmids pSU38PsfiA/*lacZ* and pSU38PsfiA**lacZ* respectively, digested with BamHI and HindIII, using the In-Fusion HD cloning kit (Clontech, Kusatsu, Japan) following manufacturer's instructions.

pPsfIAcherry-pintI1*intI*1 and pPsfIA*cherry-PintI1*intI*1 were constructed by amplifying first the *mcherry* gene encoding a red fluorescent protein from pCTX2-groE-mCherry using primers hindIII-mcherry and bamHI-mcherry2 and cloning the amplified fragment into pSU38PsfiA/*lacZ* and pSU38PsfiA**lacZ* respectively digested with BamHI and HindIII, yielding pPsfIAcherry and

pPsfIA**cherry*. Primers ter-intl-fwd-2 and inTI-pSU-rev, ter-ter-fwd-2 and ter-intl-rev-3, and pSU-ter-fwd-2 and ter-ter-rev-2, were used to amplify respectively the *intl1* gene and the lambda t0 terminator from pZE1-*intl1* (Table 1). These fragments were cloned at the XmnI site of pPsfIA*cherry* and pPsfIA**cherry* respectively using the In-Fusion HD cloning kit (Clontech). All constructs were verified by PCR and sequencing. All primers are listed in Table S1.

Time-lapse Microscopy with Microfluidic flow-cell

Experiments were performed with a CellASIC® ONIX2 microfluidic system (www.cellasic.com). Microfluidic biofilm cultures were grown in M04S microfluidic plates (EMD Millipore, Burlington, Massachusetts, USA). After priming with culture media, the cell culture chamber was loaded with 10µl of overnight bacterial culture diluted to 5X10⁷ cells/ml with a flow pressure of 0,25 psi for 6 s. Bacteria were allowed to attach to the surface for 15min without media perfusion, before starting a laminar flow of 7µl /h (1psi) for 24h. The temperature was maintained at 37°C. Image acquisition was performed with a Carl Zeiss (Oberkochen, Germany) LSM 880 inverted confocal microscope equipped with a large incubator chamber allowing temperature control, and a 40X oil immersion objective. At least three positions were selected along the chamber for real-time imaging. Images were captured every 2h during 24h with a constant 0.4 µm Z-step and were analyzed using the IMARIS 9.6 software (oxford instruments, Tubney Wood, UK). Experiments were performed at least three times.

Aerobic fluorescence recovery (AFR)

Resuspended 24 hours old microfermentor-biofilms were diluted in PBS 1X to a final concentration of 10⁷ bacteria/ml. Chloramphenicol (Cm) was added at a final concentration of

100 µg/ml to prevent further protein synthesis [26]. Samples were incubated at room temperature during 1h with shaking (300 rpm) to allow AFR [27].

Determination of the proportion of red-fluorescent bacteria by flow cytometry

The resuspend biofilm after AFR was analyzed counting 10^6 bacterial cells with a BD FACS Aria III cell sorter (Becton Dickinson, Franklin Lakes, NJ, USA). Bacteria were excited with 560nm yellow green laser and red fluorescence was collected in the PE-Texas-Red channel (610/20nm). Bacteria were gated on basis of their distribution on forward vs side scatter plots. Red bacteria were gated based on their distribution on side scatter vs PE-Texas-Red fluorescence plots. The background signal was determined with a non-fluorescent bacteria biofilm. Thresholds were applied manually to determine the boundaries between the different populations and remained the same for all experiments. Data were analyzed using the FlowLogic™ software (Inivai Technologies, Mentone, Australia).

Sorting of bacterial subpopulations

Red-fluorescent bacteria were sorted from the resuspended biofilm after AFR using a BD FACS Aria III cell sorter (Becton Dickinson). Cells were collected in Nucleoprotect RNA solution (Macherey-Nagel, Dueren, Germany). Bacteria were gated as described above. The sort collection chamber was maintained at 4°C. Up to $2 \cdot 10^6$ and $5 \cdot 10^5$ bacteria were collected in 2 hours for respectively both intermediate- and non-fluorescent-, and super-fluorescent-biofilm populations. For the *MGΔrelAΔspoT* super-fluorescent population approximately $3 \cdot 10^5$ bacteria were collected in 3 hours.

Quantification of transcripts

The pellet of sorted bacteria were incubated in 100µl of TE (30 mM Tris-HCl, 1 mM EDTA, pH 8.0) in the presence of 100µg of lysozyme and 200µg of Proteinase K for 10 min at 37°C with shaking. Total RNA was then extracted with the RNeasy Mini Kit (Qiagen, Hilden, Germany) following manufacturer's instructions. RNA concentration, quality and integrity were evaluated with the Agilent RNA 6000 Pico kit on a Bioanalyzer. cDNA were synthesized from 100 pg of RNA and quantified using the TaqMan® Fast Virus 1-Step Master Mix (ThermoFisher, Waltham, Massachusetts, USA) following manufacturer's instructions with appropriate oligonucleotides and Taqman probes (Table S1). Assays were performed with a CFX96 Touch detection system (Bio-Rad®, Hercules, Californie, USA) using the following PCR cycling conditions: 5 min at 50°C (retro-transcription reaction (RT)), 20s at 95°C (RT enzyme inactivation) and 40 cycles at 95°C for 15s and 60°C for 45s. Assays were performed in triplicate from six independent sorting.

Quantification of plasmid copy number

Sorted subpopulations were resuspended in 300 µl of PBS 1X and incubated at 95°C for 10 min. Debris were removed by centrifugation, 5 min at 11000 g. Concentration of the extracted DNA was measured with Qubit 4 Fluorometer (ThermoFisher) and normalized to 5 pg/µl in water. Quantification of *mcherry* and *gyrB* (single gene copy on the plasmid and on the chromosomal DNA respectively) copy number was made from 25 pg of DNA using the LightCycler® FastStart DNA Master HybProbe kit (Roche, Bâle, Switzerland) following manufacturer's instructions with appropriate oligonucleotides and Taqman probes (Table S1). Assays were performed with a CFX96 Touch detection system using the following PCR cycling conditions: 10 min at 95°C followed by 40 cycles at 95°C for 30s and 60°C for 1min. Assays were performed in triplicate on each population from five independent sorting. The plasmid copy number was estimated as the ratio of *mcherry* copy number/ *gyrB* copy number.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 8 (version 8.4.3) for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com. For two groups comparison a two-tailed Wilcoxon test (paired) or Mann–Whitney U-test (unpaired) test was used. Statistically significant differences were defined by a p value lower than 0.05.

Results

Heterogeneity of the SOS response level within the *E. coli* biofilm

To assess the spatial distribution of the bacteria inducing the SOS response and thus expressing *int1* within the biofilm in the absence of any exogenous stress, we used a continuous microfluidic culture system coupled with confocal microscopy allowing the development of a three-dimensional biofilm in a flow chamber and its visualization in real-time. We used the *E. coli* MG1656 F' tagged on the chromosome with the *mcherry* gene encoding a red-fluorescent protein expressed from a constitutive promoter (hereafter named MGcherry F'; Table 1). We monitored the expression of *int1* and *sfiA* genes, both regulated by the SOS response, using transcriptional fusions between their respective promoters, *Pint1* and *PsfIA*, and the fluorescent reporter gene, *gfp+*. The plasmid pPsfIA*-*gfp+*, in which the LexA binding region is mutated leading to constitutive expression of *gfp+*, was used as a positive control of fluorescence and SOS response. The pSU Δ tot/*acZ* plasmid was used as negative control (Table 1). We knew from our previous study that in biofilm, PsfIA activity fully depends on an active SOS response, as PsfIA is inactive in a MG Δ recA mutant (no activator of the SOS response, RecA) and fully derepressed in a MG Δ lexA mutant (no repressor of the SOS response, LexA) [22].

Biofilms of MGcherry F' carrying one of the plasmids were grown for 24h in LB at 37°C. As expected, the CLSM images showed that the MGcherry F'/pSU Δ tot biofilm contained only red bacteria and the MGcherry F'/pPsfiA*-*gfp*+ biofilm contained bacteria that were both red and bright green (Fig. 1a and 1b). As seen on Fig. 1c and 1d, the MGcherry F'/pPsfiA-*gfp*+ biofilm contained mainly red bacteria and very few both red and green bacteria. The 1D dot plot representing the distribution of bacteria as a function of their spatial localization further showed an uneven distribution of green bacteria on the z-axis of the 3D image of the biofilm compared to the x- and y- axes (Fig. 1g, 1e and 1f respectively). These results indicated that in the absence of exogenous stress, the *PsfiA* promoter is activated (green bacteria) in a minority of bacteria preferentially located in the depth of the biofilm.

We then estimated the proportion of these bacteria in the biofilm, using a FACS approach, which allowed to analyze each bacterium separately. To obtain enough biomass for FACS analysis, we grew our biofilms in a continuous culture in microfermentor [28]. Surprisingly, we detected no red fluorescence from the MGcherry F'/pPsfiA*-*gfp*+ biofilm, and only 87% of the bacteria in the biofilm emitted green fluorescence, which was of very low intensity. We postulated that this lack or low level of fluorescence was related to the biofilm model itself. Indeed, as the microfermentor-biofilm is thicker and larger than the microfluidic flow chamber one, the oxygen concentration might be more limiting, therefore impairing the maturation of the GFP+ and mCherry fluorescent proteins [27].

To complete the protein maturation to a fully fluorescent form, we added a step of AFR after biofilm growth [27]. The resuspended microfermentor-biofilm was incubated for one hour at room temperature with shaking in the presence of chloramphenicol to prevent further protein synthesis [26]. This method allowed full recovery of red fluorescence (mCherry) with high fluorescence emission intensity. However, for the green fluorescence, the fluorescence

intensity remained very low and too close to the background signal to be properly quantified. Therefore, the plasmid model was adjusted accordingly. We constructed PsfiA and PsfiA* transcriptional fusions with *mcherry* as the reporter gene (to follow PsfiA activity in microfermentor-biofilms by FACS). And as we were unable to visualize by CLSM *intI1* expression in the biofilm of MGcherry F'/pintI1-*gfp+*, probably due to the low level of *intI1* expression, even when the *intI1* gene is derepressed [22], we also added on the new plasmid, the *intI1* gene under the control of its own promoter PintI1, to subsequently estimate integron integrase expression by RT-qPCR (see below). These plasmids were transformed in the strain MG1656 F', leading respectively to strains MG F'/pPsfiA*cherry*-PintI1*intI1* and MG F'/pPsfiA**cherry*-PintI1*intI1* (Table 1).

After one hour of AFR, 99 % of the bacteria of a 24h-old microfermentor-biofilm of the positive control MG F'/pPsfiA**cherry*-PintI1*intI1* were bright red fluorescent (Fig. 2b). For MG F'/pPsfiA*cherry*-PintI1*intI1*, FACS dot-plot analysis showed that, as observed with the microfluidic chamber, only few bacteria expressed fluorescence in the biofilm. This approach further showed that this minority of red bacteria (3.3 ± 1.8 %) is clearly divided into two populations: one (2.3 ± 1.2 %) expressing red fluorescence at an intermediate level, the other (1 ± 0.7 %) at a high level (Fig. 2c). The latter showed red fluorescence intensity equivalent to that expressed from the constitutive promoter PsfiA*, indicating that in this population the SOS response is strongly induced, leading to full activity of the PsfiA promoter. These two red populations will be named hereafter “intermediate-” and “super-fluorescent-populations” respectively.

Differential Integron integrase expression within biofilm subpopulations

As we have previously shown that basal-expression of the *int11* and *sfiA* genes was higher in biofilm than in planktonic culture without any exogenous stress [22], we hypothesized that the expression of *int11* and *sfiA* would happen within the same subpopulations in the biofilm. We therefore sorted by FACS the three non-fluorescent, intermediate and super-fluorescent subpopulations of the MG F'/pPsfiA*cherry*-Pint11*int11* biofilm, in order to quantify in each of them the transcript levels of *int11* and *mcherry* genes (expressed from promoters Pint11 and PsfiA respectively) by RT-qPCR. First, we confirmed that no degradation of the bacterial RNA or changes in the level of *mcherry* expression occurred in the initial culture (*i.e.* re-suspended biofilm during the various steps of the experiment (AFR + sorting)) (Fig. S1). Transcript levels were higher in the intermediate population (1.9- and 1.7-fold for *cherry* and *int11* respectively) and in the super-fluorescent population (64- and 22-fold for *cherry* and *int11* respectively) than in the non-fluorescent population (Fig. 3). As the *sfiA* gene is present in the chromosome of our MG F'/pPsfiA*cherry*-Pint11*int11* strain, we therefore quantified *sfiA* transcripts to verify that its expression was also induced in the fluorescent populations of the biofilm. Surprisingly, the chromosomal *sfiA* transcript level was significantly increased but only in the super-fluorescent population and at a much lower level than that of the plasmid genes *int11* or *mcherry* (Fig. 3). Altogether, these results suggested that the strong increase in *mcherry* and *int11* transcripts may be influenced by variations in plasmid copy number in the biofilm subpopulations, in addition to the induction of the SOS response.

Influence of biofilm microniches on plasmid copy number

To answer the above questioning, we quantified the plasmid copy number in the three populations of the MG F'/pPsfiA*cherry*-Pint11*int11* biofilm. As shown on Fig. 4a, the plasmid copy number increased by 1.9- and 14.2-fold respectively in intermediate and super-

fluorescent populations compared to the non-fluorescent population. We thus also quantified the transcript level of the kanamycin resistance gene, *aph(3')-Ila*, carried on the same plasmid as the *mcherry* and *intI1* genes, and constitutively expressed. The *aph(3')-Ila* transcript level should only reflect variations in the plasmid copy number. We observed that the *aph(3')-Ila* expression level increased in both the intermediate- and super-fluorescent-populations, in proportions similar to plasmid copy number (Fig. 4b). Therefore, from now on, we normalized the transcript levels of *mcherry* and *intI1* genes by that of *aph(3')-Ila* to estimate the effect of the SOS response induction on PsfiA and Pintl1 activity. As shown on Fig. 4c, the activation of PsfiA and Pintl1 occurred only in the super-fluorescent population (by 4.1- and 1.6-fold factor respectively), in agreement with the above observations for chromosomal *sfiA* transcript levels (Fig. 3). Overall, our results show that, in the biofilm, there are two bacterial populations that express SOS-inducible genes at different levels: one expressing these genes at their low basal non-induced level (non-fluorescent and intermediate- populations), and one at a high de-repressed level (super-fluorescent population).

Induction of SOS response in the super-fluorescent population is only partially dependent on the stringent response

We previously reported that the basal expression of *sfiA* was 2.2-fold higher in biofilm than in planktonic culture and that this increase was dependent on the stringent response, which is induced by the RelA and SpoT proteins [22]. To determine whether the super-fluorescent population was specific of the biofilm and dependent on the stringent response, we analyzed by FACS a 24h-old planktonic culture of MG F'/pPsfiA*cherry*-Pintl1*intI1*, as well as a 24h-old microfermentor-biofilm of a mutant unable to induce the stringent response, MGΔ*relA*Δ*spoT* F'/pPsfiA*cherry*-Pintl1*intI1* (Table 1). Both cultures contained a super-fluorescent population

but in significantly lower proportion than in the MG F'/pPsfIAcherry-PintI1*intI1* biofilm (Fig. 5a and 5b). These results suggested that more bacteria activated the SOS-inducible promoters PintI1 and PsfIA in the biofilm than in the planktonic culture, and that in the biofilm about half of the super-fluorescent population could induce the SOS response independently of the stringent response. The next question was are the expression levels of *intI1*, *mcherry* and *sfiA* similar in all three super-fluorescent populations *i.e.* from planktonic and both biofilms (MG F' and $\Delta relA\Delta spoT$ mutant) cultures?

Complexity of the regulation of *intI1* expression by the stringent response in biofilm

The amount of super-fluorescent bacteria was too low in the planktonic culture to be sorted in a timescale suitable with our experiment. However, we were able to sort non-fluorescent and super-fluorescent populations from a 24h-old MG $\Delta relA\Delta spoT$ F'/pPsfIAcherry-PintI1*intI1* biofilms by FACS in sufficient amounts to quantify *intI1*, *mcherry* and chromosomal *sfiA* transcripts by RT-qPCR. Transcript levels from plasmidic *mcherry* and *intI1* genes were normalized to *aph(3')-IIa*, the plasmid copy number being also higher in the super-fluorescent MG $\Delta relA\Delta spoT$ F'/pPsfIAcherry-PintI1*intI1* population (Fig. S2).

In the $\Delta relA\Delta spoT$ mutant background, the transcript level of chromosomal *sfiA* was not significantly higher in the super-fluorescent population compared to the non-fluorescent population, contrary to what was observed in the wild type (WT) MG F' background (Fig. 6a), suggesting that the significant increase of *sfiA* expression in MG F'/pPsfIAcherry-PintI1*intI1* super-fluorescent population depended on the stringent response. However, the transcript levels of the plasmid genes, *mcherry* and *intI1*, were significantly higher in the MG $\Delta relA\Delta spoT$ super-fluorescent population than in the non-fluorescent population (Fig. 6b). Nevertheless, *intI1* expression ratio was still higher in the super-fluorescent population of the WT MG F'

background than in the mutant one (Fig. 6b). The trend was the same for the *mcherry* gene, although the difference of expression between the WT and mutant background super-fluorescent populations did not appear to be significant (Fig. 6b). Altogether these results indicated that for the *mcherry* and *intI1* genes, the stringent response participates but is not the only stress to be involved in the SOS response induction in the super-fluorescent population of the biofilm.

Surprisingly, although the level of *mcherry* expression was the same in the non-fluorescent WT and mutant populations, as observed for chromosomal *sfiA* expression (Fig. 6), *intI1* expression was significantly lower in the non-fluorescent mutant population than in the WT population (Fig. 6b). These results suggested that in the non-fluorescent population, the stringent response activates *intI1* expression in a SOS-independent manner.

Discussion

Bacterial biofilms are highly heterogeneous environments where bacteria experience various stress [2]. Working with the total biofilm population forces one to average and minimize the observed effects, or even miss some of them, when they occur in a small number of bacteria, as shown here. Indeed, we previously showed that the expression level of *intI1* and *sfiA* genes in the absence of exogenous stress (basal expression) was higher, but not fully de-repressed, in biofilm than in planktonic culture [22]. Here, analyzing the biofilm at the bacterial level, we showed that actually only few bacteria (around 1%) located within the depth of the biofilm activated the two promoters, *PintI1* and *PsfiA*, at a strong level. FACS analyzes were more sensitive than CLSM identifying 3 populations with different level of fluorescence (non-, intermediate- and super-fluorescent, representing in average respectively 97%, 2% and 1% of the bacteria in the biofilm; Fig. 2). However, contrary to the super-fluorescent population, in

the intermediate population, the fluorescence did not correlate with increased PsfiA and PintI1 activities, it only reflected an increase in the copy number of the ColE1-type plasmid carrying the PsfiA-*mcherry* transcriptional reporter and the integron integrase PintI1-*intI1* (Fig. 4). It has been shown that starvation/limitation of specific amino acids (Thr, Arg and Leu among those tested) led to a rise in the copy number of ColE1-type plasmids in both *relA*⁺ and *relA*⁻ *E. coli* strains. This rise was even greater in the *relA*⁻ strain than in the *relA*⁺ strain (as observed in this study, Fig. 4a and S2) for starvation of specific amino acids (Ile, Thr and His) [29]. These observations therefore confirmed that in the biofilm, bacteria undergo amino acid limitations, and further indicated that the stronger the limitation, the higher the plasmid copy number. Indeed, the super-fluorescent population which has the highest plasmid copy number is the one that undergoes a sufficiently strong amino acids limitation to induce the stringent response, which in turn would activate the SOS response [22,30]. FACS and RT-qPCR analyses also suggested the existence of other regulation pathways different from the stringent response, that could induce the SOS response, activating both PsfiA and PintI1 in the super-fluorescent population of the *MGΔrelAΔspoT* mutant biofilm (Fig. 5 and 6b). Biofilms of *E. coli ΔrelAΔspoT* mutants have been found to have reduced catalase activity (*i.e.* lower oxidative stress defense) and elevated reactive oxygen species (ROS) OH• levels [31]. ROS are known to induce the SOS response [32]. However the super-fluorescent bacteria are mainly located in the depth of the biofilm (Fig. 1) where the oxygen concentration is commonly low [2]. Therefore, the likelihood that oxidative stress *via* ROS production is one of the signals that induce the SOS response in the super-fluorescent population of *ΔrelAΔspoT* mutant biofilm does not seem very likely, but would need to be investigated. Unfortunately, we could not verify this hypothesis as we were not able to construct an *MGcherryΔrelAΔspoT* F' mutant strain to monitor and localize the PsfiA-*gfp*⁺ expression in flow-cell, as we did with the WT strain

(Fig. 1). Another stress inducing the SOS response in the biofilm, could be phosphate starvation, which has also been found to induce the LexA regulon [33].

We were surprised to find that the level of *intI1* expression in the non-fluorescent $\Delta relA\Delta spoT$ population was significantly lower than in the non-fluorescent WT MG F' population (Fig. 6b).

It indeed suggested that the basal activity of *PintI1* is reduced in the non-fluorescent population in the absence of (p)ppGpp (alarmones produced by RelA and SpoT upon nutrient starvation). Since this effect was not observed for the *PsfIA* promoter, it appears to be specific to *PintI1* and independent of the SOS response. Interestingly, Strugeon *et al.* have shown that in biofilm, the expression of *PintI1** (*PintI1* promoter with a mutated LexA-binding-site) is regulated by the Lon protease, which was not the case with WT *PintI1* [22]. The Lon protease is known to be activated by the polyphosphate (poly-P) that accumulates upon the stringent response induction. In the model proposed by Strugeon *et al.*, the poly-P-Lon complex would control the stability of an unidentified regulator of *PintI1* (repressor) that would only act when the *PintI1* promoter is released from LexA. Thus, one could imagine that in the non-fluorescent population of the $\Delta relA\Delta spoT$ mutant biofilm, the basal level of poly-P-Lon is no longer sufficient to ensure the degradation of the inhibitor of the derepressed *PintI1* promoter. In this case, the basal activity of *PintI1* would decrease due to the absence of (p)ppGpp and the lower level of poly-P-Lon. Another hypothesis to explain this decrease in basal *PintI1* activity in the $\Delta relA\Delta spoT$ non-fluorescent population would be a direct effect on the transcription. Indeed, (p)ppGpp regulates positively or negatively transcription initiation from specific promoters by binding directly to RNA polymerase (RNAP) at two sites [34,35]. In these (p)ppGpp regulated promoters, a discriminator region (region between the conserved -10 promoter element and the transcription start site (TSS)) with more A+T rich content was linked to activated promoters [35,36]. *PintI1* shares some characteristics of (p)ppGpp activated

promoters (conserved bases in the -10 region, T and A bases in the discriminator region and TSS [35,37]). It is tempting to assume that the *intI1* promoter basal activity in the biofilm might be stimulated by (p)ppGpp, but this will need to be confirmed.

Overall, our results showed that the regulation of integron integrase expression in biofilm is complex. There are two main populations with different characteristics in the biofilm: the vast majority of the bacteria expressing the SOS-regulon gene *sfiA* and *intI1* at a basal level, which for *intI1* expression seems to depend on (p)ppGpp; and a minor population (1%), located in the depth of the biofilm that faces amino acid limitations leading to the induction of the stringent response, as well as other limitations (phosphate ?), all being endogenous stresses inducing the activation of SOS-regulated promoters in this population (Fig. S3). As a result, this population would be able to acquire antibiotic resistance genes *via* the expression of *IntI1*.

Dorr *et al.* showed that the formation of *E. coli* persisters (subpopulation of bacteria (usually less than 1%) surviving in presence of high bactericidal antibiotic pressure without an increase in MIC [38,39]) was induced by the SOS response after fluoroquinolone treatment [40]. Bernier *et al.* demonstrated that the tolerance of *E. coli* biofilm to ofloxacin depended on starvation and SOS response [41]. Intriguingly, our super-fluorescent-biofilm population shares several characteristics describing triggered persistence: i) it is induced upon amino acids limitation (via the stringent response) and possibly other nutrients limitations, ii) it represents a minor proportion (around 1%) of the biofilm and iii) both states (super-fluorescence/SOS induction and persistence) are transient. Indeed, persistent bacteria, when treated with bactericidal antibiotics, will regrow when diluted in an antibiotic-free media, their progeny being as sensitive to drugs as the parental population. And, in our case, when grown again in microfermentor, sorted super-fluorescent bacteria of the MG F'/Psf*iA*cherry-PintI1*intI1* biofilm gave rise to a new biofilm containing the same sub-populations as in the

initial biofilm (Fig. S4). It is tempting to infer that the super-fluorescent-biofilm population could be a persister population induced by endogenous stresses of the biofilm. If so, it would mean that in the biofilm, the super-fluorescent population, which expresses the SOS response at high level, would be both tolerant to antibiotics and ready to express antibiotic resistance through gene cassettes acquisition or shuffling via the integron integrase. Furthermore, the activation of the SOS response in the super-fluorescent population could also induce error-prone DNA polymerases leading to mutations involved in antibiotic resistance (Fig. S3). In the case of antibiotic treatment, these features would allow resistant bacteria to be readily selected for. It was indeed recently shown that persisters of *E. coli* after fluoroquinolone treatment displayed enhanced resistance toward several antibiotics following recovery from treatment, and this was linked to the SOS response and the error-prone polymerase V [42].

It has been long supposed in clinical practice, that the main characteristic hampering biofilm treatment was their thick-layered structure, limiting the penetration of antibiotics, thus exposing them to sub-MIC concentrations that would facilitate the selection of antimicrobial resistance. Nowadays, it is clear that biofilm endurance is multifactorial [13,43]. It was shown in a *P. aeruginosa* biofilm that some bacteria undergo endogenous oxidative stress leading to double-strand DNA breaks. The mechanisms involved in repair of these DNA breaks, which were SOS-response-independent, generated genetic variations within the population, conferring various selective advantages, in particular mutations that increased the emergence of antibiotic resistant bacteria that could be selected for when biofilms were exposed to antibiotic treatment [44]. Here we demonstrate, that in addition to its structural advantage, the biofilm lifestyle promotes levels of local endogenous stresses, even in the absence of antibiotic pressure, that give rise to a small population of bacteria expressing at high-level, genes regulated by the SOS response. This population which shows most of the characteristics

required for tolerance to antibiotics and for antibiotic resistance acquisition, could constitute a reservoir of bacteria fully ready to rapidly resist antibiotic treatment, being the cause of recurrent infections and impacting the management of nosocomial infections and antibiotic treatments.

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Conflict of interest

The authors declare no conflict of interest.

References

- [1] Monds RD, O'Toole GA. The developmental model of microbial biofilms: ten years of a paradigm up for review. *Trends in Microbiology* 2009;17:73–87. <https://doi.org/10.1016/j.tim.2008.11.001>.
- [2] Stewart PS, Franklin MJ. Physiological heterogeneity in biofilms. *Nature Reviews Microbiology* 2008;6:199–210. <https://doi.org/10.1038/nrmicro1838>.
- [3] An D, Parsek MR. The promise and peril of transcriptional profiling in biofilm communities. *Current Opinion in Microbiology* 2007;10:292–6. <https://doi.org/10.1016/j.mib.2007.05.011>.
- [4] Bagge N, Hentzer M, Andersen JB, Ciofu O, Givskov M, Høiby N. Dynamics and spatial distribution of beta-lactamase expression in *Pseudomonas aeruginosa* biofilms. *Antimicrob Agents Chemother* 2004;48:1168–74. <https://doi.org/10.1128/aac.48.4.1168-1174.2004>.
- [5] Ito A, May T, Taniuchi A, Kawata K, Okabe S. Localized expression profiles of *rpoS* in *Escherichia coli* biofilms. *Biotechnology and Bioengineering* 2009;103:975–83. <https://doi.org/10.1002/bit.22305>.
- [6] Moormeier DE, Endres JL, Mann EE, Sadykov MR, Horswill AR, Rice KC, et al. Use of Microfluidic Technology To Analyze Gene Expression during *Staphylococcus aureus* Biofilm Formation Reveals Distinct Physiological Niches. *Appl Environ Microbiol* 2013;79:3413–24. <https://doi.org/10.1128/AEM.00395-13>.
- [7] Pabst B, Pitts B, Lauchnor E, Stewart PS. Gel-Entrapped *Staphylococcus aureus* Bacteria as Models of Biofilm Infection Exhibit Growth in Dense Aggregates, Oxygen Limitation, Antibiotic Tolerance, and Heterogeneous Gene Expression. *Antimicrob Agents Chemother* 2016;60:6294–301. <https://doi.org/10.1128/AAC.01336-16>.
- [8] Zaborskyte G, Andersen JB, Kragh KN, Ciofu O. Real-Time Monitoring of *nfxB* Mutant Occurrence and Dynamics in *Pseudomonas aeruginosa* Biofilm Exposed to Subinhibitory Concentrations of

479 Ciprofloxacin. *Antimicrob Agents Chemother* 2017;61. <https://doi.org/10.1128/AAC.02292-16>.

480 [9] Ambriz-Aviña V, Contreras-Garduño JA, Pedraza-Reyes M. Applications of Flow Cytometry to
481 Characterize Bacterial Physiological Responses. *Biomed Res Int* 2014;2014.
482 <https://doi.org/10.1155/2014/461941>.

483 [10] Garcia-Betancur JC, Yepes A, Schneider J, Lopez D. Single-cell Analysis of *Bacillus subtilis* Biofilms
484 Using Fluorescence Microscopy and Flow Cytometry. *J Vis Exp* 2012. <https://doi.org/10.3791/3796>.

485 [11] Marlow VL, Cianfanelli FR, Porter M, Cairns LS, Dale JK, Stanley-Wall NR. The prevalence and
486 origin of exoprotease-producing cells in the *Bacillus subtilis* biofilm. *Microbiology* 2014;160:56–66.
487 <https://doi.org/10.1099/mic.0.072389-0>.

488 [12] Madsen JS, Burmølle M, Hansen LH, Sørensen SJ. The interconnection between biofilm
489 formation and horizontal gene transfer. *FEMS Immunology & Medical Microbiology* 2012;65:183–95.
490 <https://doi.org/10.1111/j.1574-695X.2012.00960.x>.

491 [13] Lebeaux D, Ghigo J-M, Beloin C. Biofilm-Related Infections: Bridging the Gap between Clinical
492 Management and Fundamental Aspects of Recalcitrance toward Antibiotics. *Microbiology and*
493 *Molecular Biology Reviews* 2014;78:510–43. <https://doi.org/10.1128/MMBR.00013-14>.

494 [14] Gillings MR. Integrins: past, present, and future. *Microbiol Mol Biol Rev* 2014;78:257–77.
495 <https://doi.org/10.1128/MMBR.00056-13>.

496 [15] Lacotte Y, Ploy M-C, Raherison S. Class 1 integrins are low-cost structures in *Escherichia coli*.
497 *ISME J* 2017;11:1535–44. <https://doi.org/10.1038/ismej.2017.38>.

498 [16] Stokes HW, Hall RM. A novel family of potentially mobile DNA elements encoding site-specific
499 gene-integration functions: integrins. *Molecular Microbiology* 1989;3:1669–83.
500 <https://doi.org/10.1111/j.1365-2958.1989.tb00153.x>.

501 [17] Collis CM, Hall RM. Site-specific deletion and rearrangement of integron insert genes catalyzed

502 by the integron DNA integrase. J Bacteriol 1992;174:1574–85.

503 [18] Escudero* JA, Loot* C, Nivina A, Mazel D. The Integron: Adaptation On Demand. Microbiology
504 Spectrum 2015;3. <https://doi.org/10.1128/microbiolspec.MDNA3-0019-2014>.

505 [19] Labbate M, Case RJ, Stokes HW. The Integron/Gene Cassette System: An Active Player in
506 Bacterial Adaptation. In: Gogarten MB, Gogarten JP, Olendzenski LC, editors. Horizontal Gene
507 Transfer: Genomes in Flux, Totowa, NJ: Humana Press; 2009, p. 103–25.
508 https://doi.org/10.1007/978-1-60327-853-9_6.

509 [20] Guerin É, Cambray G, Sanchez-Alberola N, Campoy S, Erill I, Re SD, et al. The SOS Response
510 Controls Integron Recombination. Science 2009;324:1034–1034.
511 <https://doi.org/10.1126/science.1172914>.

512 [21] Baharoglu Z, Mazel D. SOS, the formidable strategy of bacteria against aggressions. FEMS
513 Microbiology Reviews 2014;38:1126. <https://doi.org/10.1111/1574-6976.12077>.

514 [22] Strugeon E, Tilloy V, Ploy M-C, Re SD. The Stringent Response Promotes Antibiotic Resistance
515 Dissemination by Regulating Integron Integrase Expression in Biofilms. MBio 2016;7:e00868-16.
516 <https://doi.org/10.1128/mBio.00868-16>.

517 [23] Cashel M, Gentry DR, Hernandez VH, Vinella D. The stringent response. In: Neidhardt F. C, Curtis
518 III R, Ingraham J. L, Lin E. C.C, Low KB, Magasanik B, et al., editors. *Escherichia coli* and *Salmonella*:
519 cellular and molecular biology. 2nd ed., ASM press; 1996.

520 [24] Huang C-T, Xu KD, McFeters GA, Stewart PS. Spatial Patterns of Alkaline Phosphatase Expression
521 within Bacterial Colonies and Biofilms in Response to Phosphate Starvation. Appl Environ Microbiol
522 1998;64:1526–31.

523 [25] Datsenko KA, Wanner BL. One-step inactivation of chromosomal genes in *Escherichia coli* K-12
524 using PCR products. Proc Natl Acad Sci U S A 2000;97:6640–5.
525 <https://doi.org/10.1073/pnas.120163297>.

526 [26] Andrews JM. Determination of minimum inhibitory concentrations. J Antimicrob Chemother
527 2001;48:5–16. https://doi.org/10.1093/jac/48.suppl_1.5.

528 [27] Pinilla-Redondo R, Riber L, Sørensen SJ. Fluorescence Recovery Allows the Implementation of a
529 Fluorescence Reporter Gene Platform Applicable for the Detection and Quantification of Horizontal
530 Gene Transfer in Anoxic Environments. Appl Environ Microbiol 2018;84.
531 <https://doi.org/10.1128/AEM.02507-17>.

532 [28] Ghigo J-M. Natural conjugative plasmids induce bacterial biofilm development. Nature
533 2001;412:442–5. <https://doi.org/10.1038/35086581>.

534 [29] Wrobel B, Wegrzyn G. Differential amplification efficiency of pMB1 and p15A (ColE1-type)
535 replicons in *Escherichia coli* stringent and relaxed strains starved for particular amino acids.
536 Microbiological Research 1997;152:251–5. [https://doi.org/10.1016/S0944-5013\(97\)80036-5](https://doi.org/10.1016/S0944-5013(97)80036-5).

537 [30] Durfee T, Hansen AM, Zhi H, Blattner FR, Jin DJ. Transcription profiling of the stringent response
538 in *Escherichia coli*. J Bacteriol 2008;190:1084–96. <https://doi.org/10.1128/JB.01092-07>.

539 [31] Nguyen D, Joshi-Datar A, Lepine F, Bauerle E, Olakanmi O, Beer K, et al. Active starvation
540 responses mediate antibiotic tolerance in biofilms and nutrient-limited bacteria. Science
541 2011;334:982–6. <https://doi.org/10.1126/science.1211037>.

542 [32] Imlay JA, Linn S. Mutagenesis and stress responses induced in *Escherichia coli* by hydrogen
543 peroxide. J Bacteriol 1987;169:2967–76. <https://doi.org/10.1128/jb.169.7.2967-2976.1987>.

544 [33] Dri AM, Moreau PL. Phosphate starvation and low temperature as well as ultraviolet irradiation
545 transcriptionally induce the *Escherichia coli* LexA-controlled gene *sfiA*. Mol Microbiol 1993;8:697–
546 706. <https://doi.org/10.1111/j.1365-2958.1993.tb01613.x>.

547 [34] Gourse RL, Chen AY, Gopalkrishnan S, Sanchez-Vazquez P, Myers A, Ross W. Transcriptional
548 Responses to ppGpp and DksA. Annu Rev Microbiol 2018;72:163–84.
549 <https://doi.org/10.1146/annurev-micro-090817-062444>.

- 550 [35] Sanchez-Vazquez P, Dewey CN, Kitten N, Ross W, Gourse RL. Genome-wide effects on
551 *Escherichia coli* transcription from ppGpp binding to its two sites on RNA polymerase. Proc Natl Acad
552 Sci U S A 2019;116:8310–9. <https://doi.org/10.1073/pnas.1819682116>.
- 553 [36] Cabrer-Panes JD, Fernández-Coll L, Fernández-Vázquez J, Gaviria-Cantin TC, Mouali YE, Åberg A,
554 et al. ppGpp mediates the growth phase-dependent regulation of *agn43*, a phase variable gene, by
555 stimulating its promoter activity. Environmental Microbiology Reports 2020;12:444–53.
556 <https://doi.org/10.1111/1758-2229.12860>.
- 557 [37] Guerin E, Jove T, Tabesse A, Mazel D, Ploy MC. High-level gene cassette transcription prevents
558 integrase expression in class 1 integrons. Journal of Bacteriology 2011;193:5675–82.
559 <https://doi.org/10.1128/JB.05246-11>.
- 560 [38] Balaban NQ, Helaine S, Lewis K, Ackermann M, Aldridge B, Andersson DI, et al. Definitions and
561 guidelines for research on antibiotic persistence. Nat Rev Microbiol 2019;17:441–8.
562 <https://doi.org/10.1038/s41579-019-0196-3>.
- 563 [39] Brauner A, Fridman O, Gefen O, Balaban NQ. Distinguishing between resistance, tolerance and
564 persistence to antibiotic treatment. Nat Rev Microbiol 2016;14:320–30.
565 <https://doi.org/10.1038/nrmicro.2016.34>.
- 566 [40] Dörr T, Lewis K, Vulić M. SOS Response Induces Persistence to Fluoroquinolones in *Escherichia*
567 *coli*. PLoS Genet 2009;5:e1000760. <https://doi.org/10.1371/journal.pgen.1000760>.
- 568 [41] Bernier SP, Lebeaux D, DeFrancesco AS, Valomon A, Soubigou G, Coppée J-Y, et al. Starvation,
569 together with the SOS response, mediates high biofilm-specific tolerance to the fluoroquinolone
570 ofloxacin. PLoS Genet 2013;9:e1003144. <https://doi.org/10.1371/journal.pgen.1003144>.
- 571 [42] Barrett TC, Mok WWK, Murawski AM, Brynildsen MP. Enhanced antibiotic resistance
572 development from fluoroquinolone persisters after a single exposure to antibiotic. Nat Commun
573 2019;10:1177. <https://doi.org/10.1038/s41467-019-09058-4>.

574 [43] Hall CW, Mah T-F. Molecular mechanisms of biofilm-based antibiotic resistance and tolerance in
575 pathogenic bacteria. *FEMS Microbiology Reviews* 2017;41:276–301.
576 <https://doi.org/10.1093/femsre/fux010>.

577 [44] Boles BR, Singh PK. Endogenous oxidative stress produces diversity and adaptability in biofilm
578 communities. *Proceedings of the National Academy of Sciences* 2008;105:12503–8.
579 <https://doi.org/10.1073/pnas.0801499105>.

580 [45] Espéli O, Moulin L, Boccard F. Transcription attenuation associated with bacterial repetitive
581 extragenic BIME elements. *J Mol Biol* 2001;314:375–86. <https://doi.org/10.1006/jmbi.2001.5150>.

582 [46] Beloin C, Valle J, Latour-Lambert P, Faure P, Kzreminski M, Balestrino D, et al. Global impact of
583 mature biofilm lifestyle on *Escherichia coli* K-12 gene expression. *Molecular Microbiology*
584 2004;51:659–74. <https://doi.org/10.1046/j.1365-2958.2003.03865.x>.

585 [47] Chaveroche MK, Ghigo JM, d’Enfert C. A rapid method for efficient gene replacement in the
586 filamentous fungus *Aspergillus nidulans*. *Nucleic Acids Res* 2000;28:E97.
587 <https://doi.org/10.1093/nar/28.22.e97>.

588 [48] Cherepanov P, Wackernagel W. Gene disruption in *Escherichia coli*: TcR and KmR cassettes with
589 the option of Flp-catalyzed excision of the antibiotic-resistance determinant. *Gene* 1995;158:9–14.
590 [https://doi.org/10.1016/0378-1119\(95\)00193-A](https://doi.org/10.1016/0378-1119(95)00193-A).

591 [49] Jové T, Da Re S, Denis F, Mazel D, Ploy M-C. Inverse Correlation between Promoter Strength and
592 Excision Activity in Class 1 Integrations. *PLoS Genet* 2010;6.
593 <https://doi.org/10.1371/journal.pgen.1000793>.

594 [50] Cisz M, Lee P-C, Rietsch A. ExoS Controls the Cell Contact-Mediated Switch to Effector Secretion
595 in *Pseudomonas aeruginosa*. *Journal of Bacteriology* 2008;190:2726–38.
596 <https://doi.org/10.1128/JB.01553-07>.

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Fig. 1: *sfiA* expression is heterogeneous and localized within the biofilm

a, b, c, CLSM images and section of the XZ planes of respectively MGcherry F'/pSUDtot, MGcherry F'/pPsfiA*-gfp+ and MGcherry F'/pPsfiA-gfp+ biofilms grown for 24h at 37°C in microfluidic flow-cell chamber. **d,** XY plan section of the MGcherry F'/pPsfiA-gfp+ biofilm image (c) at 5 μm depth. z-stacks were generated using a Zeiss LSM 880 microscope and processed with Imaris 9.6 software (Bitplane). Scale bar represents 30 μm. **e, f, g,** 1D plot representing the total number of green and red fluorescent bacteria in the biofilm as a function of their X-position, Y-position and Z-position respectively. Boxplots are designed as median, the lower quartile (Q1) and the upper quartile (Q3). Whiskers are extended to the extreme data points. All images are representative of one position among 10 taken for each condition. Experiments were done in triplicate.

Fig. 2. Quantification of cells expressing *sfIA* in biofilm

Flow cytometry dot-plot analyses of a 24h-old biofilm of strain (a) MG F'/pSU Δ tot, (b) MG F'/pPsfIA**cherry*-Pint1*int1* and (c) MG F'/pPsfIA*cherry*-Pint1*int1* grown in continuous culture in microfermentor. Counting was performed after one hour of aerobic fluorescence recovery at room temperature. The black square delimits the cherry-positive population (cherry +) based on the non-fluorescent strain MG F'/pSU Δ tot (panel a), and the red rectangle defines the cherry-super-fluorescent population (super Fluo) based on derepressed cherry expression in strain MG F'/ pPsfIA**cherry*-Pint1*int1* (panel b). The data represent one representative experimental replicate.

Fig. 3. Differential expression of *mcherry*, *intl1* and *sfiA* in biofilm subpopulations

Expression ratio of *mcherry*, *intl1* and *sfiA* transcripts from initial culture (dark pink) and sorted sub-populations (non-fluorescent (light pink), intermediate (medium pink) and super-fluorescent (red)) of a 24-old biofilm of MG F' pPsfACherry-Pint1*intl1* grown in microfermentor. Relative quantification of *mcherry*, *intl1* and *sfiA* was performed using the $2^{-\Delta\Delta C_t}$ method, the transcript number of each gene was normalized to the endogenous *gyrB* gene and calibrated to the non-fluorescent population. Data are the average of transcripts levels measured from 6 independent sorting experiments. Errors bars indicate the SD. Asterisks indicate significant difference: ** $p < 0.01$; ns: non-significant (Wilcoxon test).

Fig. 4: Impact of the variation in plasmid copy number in the biofilm sub-populations on *mcherry* and *int11* expression level

a, The plasmid copy number was estimated by calculating the ratio of gene copies number of plasmidic *mcherry* over chromosomal *gyrB* for each sorted sub-populations from a 24h-old biofilm of MG F' pPsfiA*cherry*-Pint1*int11* strain grown in microfermentor. **b**, Expression ratio of *aph(3')-IIa* transcripts from sorted sub-populations NF, inter and super + (same biofilm as in a). Relative quantification of *aph(3')-II* (gene encoding kanamycin resistance carried on the same plasmid as *mcherry*) was performed using the $2^{-\Delta\Delta C_t}$ method, with normalization to the endogenous *gyrB* gene and calibration to the non-fluorescent population. **c**, Expression ratio of *mcherry* and *int11* transcripts in sorted sub-populations, NF, inter and super +. Relative quantification of *mcherry* and *int11* was performed using the $2^{-\Delta\Delta C_t}$ method, with normalization to the *aph(3')-IIa* gene and calibration to the non-fluorescent population. NF (non-fluorescent; light pink), inter (intermediate; medium pink) and SF (super-fluorescent; red) sub-populations. Data are the average of gene copies number or transcripts levels measured from 6 independent sorting experiments: Average and standard deviation are shown as black lines (a and c), errors bars indicate the SD (b). Asterisks indicate significant difference: * $p < 0.05$; **** $p < 0.0001$; ns: non-significant (Wilcoxon test).

Fig. 5: Comparison of cherry expression level in biofilm and planktonic conditions, and effect of the stringent response on mcherry expression in biofilm

Proportion (%) of cherry-super-fluorescent populations in (a) biofilm (B, orange) and planktonic (P, blue) cultures of the MG F'/pPsfACherry-Pint1int1 strain; (b) in biofilm of MG F' and MGΔrelAΔspoT F' strains carrying plasmid pPsfACherry-Pint1int1. Data are the average of at least 12 independent cultures. Asterisks indicate significant difference between biofilm and planktonic conditions (a) or between MG and MGΔrelAΔspoT (b); *** $p < 0.001$ (Mann-Whitney U-test (a) or Wilcoxon test (b)). Average and standard deviation are shown as black lines.

Fig. 6: The stringent response induces the expression of *sfiA*, *mcherry* and *int11* in the biofilm super-fluorescent subpopulations.

Expression ratio of (a) *sfiA*, (b) *mcherry* and *int11* transcripts number in non-fluorescent (NF; light pink) and super-fluorescent (SF; red) sorted sub-populations of 24-old biofilms of MG F' (circle) and *MGΔrelAΔspoT* F' (triangle) strains carrying pPsfiA*cherry*-Pint1*int11*. Relative quantification of *mcherry*, *int11* and *sfiA* was performed using the $2^{-\Delta\Delta C_t}$ method. The number of *mcherry* and *int11* transcripts was normalized to that of *Kana* gene (constitutively expressed from the plasmid) to take in to account plasmid number variation; the transcripts number of chromosomal *sfiA* gene was normalized to the endogenous *gyrB* gene. The calibrator was MG F' pPsfiA*cherry*-Pint1*int11* non-fluorescent sub-population. Data are the average of transcripts levels measured from at least 6 independent sorting experiments. *** $p < 0.001$; ** $p < 0.01$; ns: non-significant; Mann-Whitney U-test (comparison of WT MG F' vs *MGΔrelAΔspoT* F') or Wilcoxon test (comparison of NF and SF populations of the same biofilm). Average and standard deviation are shown as black lines.

674 **Table 1: Strains and plasmids used in this study**

Strains and plasmids	Genotype or description	Reference or use
<i>E. coli</i> Strains		
MG1656 F'	<i>lacZ</i> null derivative of <i>E. coli</i> MG1655 carrying the F' plasmid	[45]
NEB 5-alpha F= Iq	F= <i>proA_B_lacIq_(lacZ)</i> M15 <i>zzf::TnI0</i> (Tetr)/ <i>fhuA2_(argF-lacZ)</i> U169 <i>phoA glnV44</i> _80_(<i>lacZ</i>)M15 <i>gyrA96 recA1 relA1 endA1 thi-1</i> <i>hsdR17</i>	New England Biolabs, Evry, France
MG1656Δ <i>cpxI</i> ::km-frt	<i>cpxI</i> gene replaced by the km-frt cassette in strain MG1655	[46]
MG1656λatt::mcherry F'	Insertion, at the λatt site, of the <i>mcherry</i> gene under the control of the constitutive λpR promoter	This study
MG1656Δ <i>relA</i> Δ <i>spoT</i> F'	Deletion of <i>relA</i> and <i>spoT</i> in MG1656	[22]
Plasmids		
pKOBEGA	λ-red recombination system expression plasmid	[47]
pCP20	Vector carrying the <i>flp</i> gene encoding the FLP recombinase specific to FRT sites, thermosensitive; Amp ^r Cm ^r	[48]
pZE1- <i>intI1</i>	<i>intI1</i> gene under the control of its own promoter; Amp ^r	This study
pSU38Δ <i>tot</i> / <i>lacZ</i>	Vector carrying the <i>lacZ</i> coding sequence with no	[49]

	translation initiation region nor promoter; Km ^r	
pSU38PsfiA/ <i>lacZ</i>	<i>sfiA</i> promoter cloned into pSU38Δ <i>tot</i> / <i>lacZ</i> ; <i>lacZ</i> under the control of PsfiA; Km ^r	[22]
pSU38PsfiA* <i>lacZ</i>	<i>sfiA</i> promoter cloned into pSU38Δ <i>tot</i> / <i>lacZ</i> ; <i>lacZ</i> under the control of PsfiA*, PsfiA carrying the mutation LexAmut2 in the LexA box, inhibiting LexA binding and leading to constitutive expression [20]; Km ^r	Laboratory collection
F'	F' conjugative plasmid allowing enhanced biofilm formation; Tet ^R	[28]
pPsfiA- <i>gfp+</i>	<i>gfp+</i> under the control of the promoter of the <i>sfiA</i> gene, PsfiA; Km ^r	This study
pPsfiA*- <i>gfp+</i>	<i>gfp+</i> under the control of PsfiA*; Km ^r	This study
pCTX2- <i>groE</i> -mCherry	p <i>groE</i> controlling mCherry expression	[50]
pPsfiA <i>cherry</i> -PintI1 <i>intI1</i>	<i>mcherry</i> under the control of PsfiA, and <i>intI1</i> from In40 class I integron under the control of its own promoter PintI1; Km ^r	This study
pPsfiA* <i>cherry</i> -PintI1 <i>intI1</i>	pPsfiA <i>cherry</i> -PintI1 <i>intI1</i> carrying the mutation LexAmut2 in the LexA box of PsfiA; Km ^r	This study
pGZA1	pUC18-mini Tn7T-Gm-P _{CD} - <i>gfp+</i> ; Gen ^r , amplification matrix for the <i>gfp+</i> gene	[8]