

## SUPPLEMENTAL INFORMATION

### Evolution of contact-dependent inhibition in non-growing populations of *E. coli*

Marc Lemonnier<sup>1,2,3</sup>, Bruce R. Levin<sup>3,\*</sup>, Tony Romeo<sup>4</sup>, Kim Garner,<sup>3</sup> María-Rosario Baquero<sup>5</sup>, Jeff Mercante<sup>4</sup>, Emmanuel Lemichez<sup>2</sup>, Fernando Baquero<sup>5</sup>, and Jesús Blázquez<sup>5,6</sup>

<sup>1</sup>Department of Molecular Microbiology, Centro de Investigaciones Biológicas CSIC, Madrid, Spain.

<sup>2</sup>INSERM, U627, Faculté de Médecine, Nice, France.

<sup>3</sup>Department of Biology, Emory University, Atlanta, GA, USA.

<sup>4</sup>Department of Microbiology and Immunology, Emory University School of Medicine, Atlanta, GA, USA.

<sup>5</sup>Department of Microbiology, Ramón y Cajal Hospital, Madrid, Spain.

<sup>6</sup>Departamento de Biotecnología Microbiana, Centro Nacional de Biotecnología, Madrid Spain

\*Corresponding Author: [blevin@emory.edu](mailto:blevin@emory.edu)

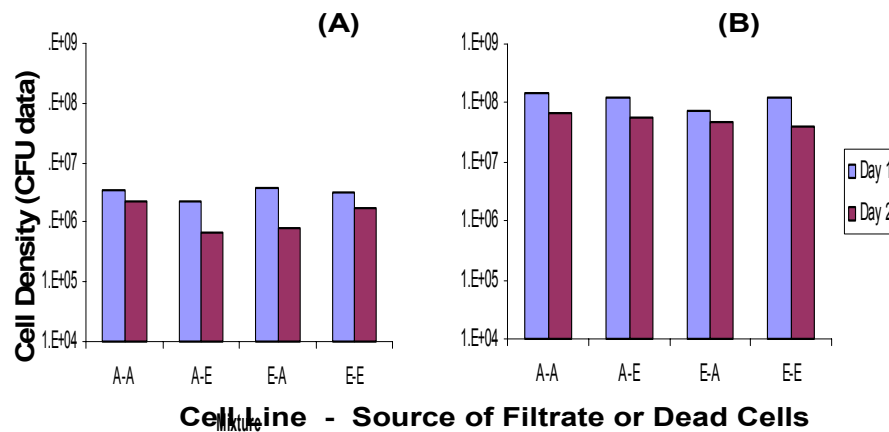
## CONTENTS

Supplementary Results (including their specific experimental procedures).....	
1. Additional evidence for contact dependence.....	page 2
2. The SCDI phenomenon and bacteria.....	page 3
3. The genetic basis of SCDI and immunity to SCDI in <i>E. coli</i> ABM.....	page 7
4. Glycogen production and SCDI .....	page 11
5. Cell viability analysis during SCDI using confocal microscopy/ SCDI is not due to plating efficiency differences .....	page 14
6. Evidence for SCDI with other strains of <i>E. coli</i> .....	page 20
7. Population and Evolutionary Dynamics of SCDI.....	page 22

## SUPPLEMENTARY RESULTS

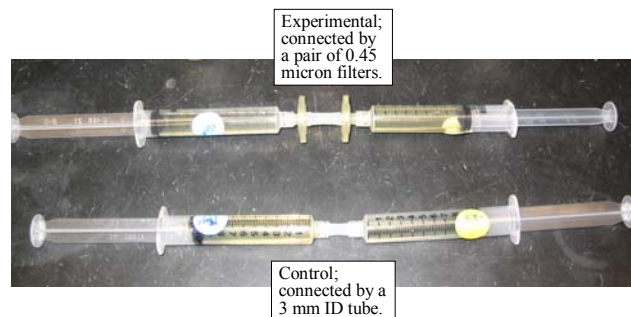
### 1- Additional Evidence for contact dependence:

In addition to the FACS experiments considered in our report, two other lines of evidence support the hypothesis that the inhibition of growth of the ancestral ABM strains requires physical contact with viable evolved *glgC* mutants. One is that the rates of decline in the estimated density of ancestral cells are effectively the same when they are in sterile filtrates or sonicated or heat killed suspensions of stationary phase cultures of the evolved cells or their own cells. The results of two of the experiments providing this evidence are presented in Figure S1. In these experiments stationary phase cultures of ancestral (A) or evolved (E) cells were incubated with shaking at 37°C in stationary phase filtrates or suspensions of heat-killed stationary phase cells. The densities of ancestral and evolved cells in these media were estimated from CFU after one and two days of incubation.



**Figure S1. Survival of stationary phase ancestral and evolved cells in sterile filtrates or cultures of heat killed stationary phase ancestral or evolved cells. A-A ancestral in filtrates or suspensions of dead ancestral cells; A-E ancestral cells in filtrates or suspensions of dead evolved cells; E-A evolved cells in filtrates or suspensions of dead ancestral cells; E-E evolved cells in filtrates of suspensions of heat-killed evolved! cells. (A) Sterile filtrates, (B) Suspensions of heat-killed (CFU <math>10^4</math> viable) cells.**

The second line of additional evidence for cell-cell contact being required for the observed inhibition comes from experiments with variant of the classic Davis U-tube. In this case the “U-tubes” were pairs of 10 ml plastic syringes connected either directly with silicon tubes or through pairs of 0.45 micron filter (Figure S2). The ancestral and evolved cultures were put into opposite syringes and mixed by forcing the plungers at least seven times during a 24 hour periods. In between mixing they were held vertically, shaken and plugged to allow for air to enter,



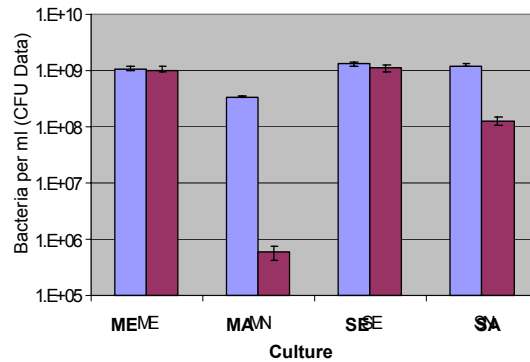
**Figure S2: Make-shift “U-tube” used to test for contact-dependence**

The initial densities of ancestral (Ara<sup>+</sup>) and evolved (Ara<sup>-</sup>) cells in the filter separated experimental, and control syringes were  $9.8 \times 10^8$  and  $7.4 \times 10^8$ , respectively for an Evolved/Ancestral (E/A) ratio of 0.76. At 24 hours, in the experimental syringes the mean number of bacteria from two samples taken from the syringes carrying the ancestral and evolved cells were,  $4.1 \times 10^8$  and  $5.5 \times 10^8$  respectively for an evolved to ancestral ratio of 1.3. In the control syringes where the cells were mixed this ratio was 300.6.

## **2- The SCDI Phenomenon and bacteria**

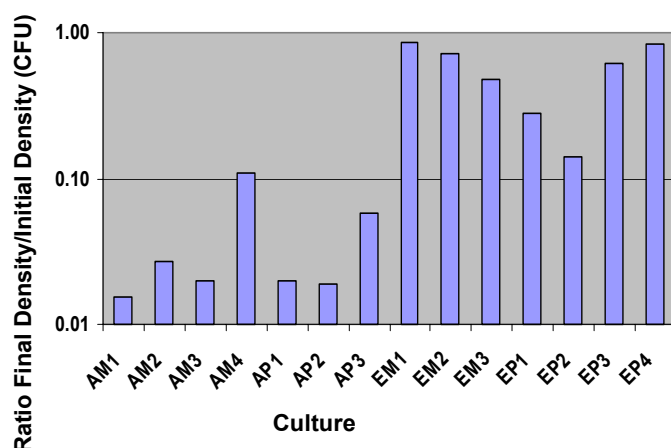
**(i) Variability:** In the course of this investigation, we performed more than 150 mixed culture experiments similar to those reported in Figure 1 with the ancestral Ara<sup>-</sup> or Ara<sup>+</sup> ABM bacteria and different separately evolved Ara<sup>+</sup> and Ara<sup>-</sup> bacteria at high densities

with evolved cells in frequencies in excess of 0.1. Quantitatively SCDI is a somewhat variable process; the extent to which the ancestral cells were inhibited by the evolved varied between experiments with the same pairs of ancestral ABM and evolved as well with Ara<sup>+</sup> and Ara<sup>-</sup> ancestral cells with independently evolved ABM. In growing LB culture initiated with approximately equal frequencies of ancestral and evolved cells, the decline in the density of ancestral population varied from 200 fold to more than 5000 fold. Within a particular experiment, there is relatively little variability in the extent of inhibition of the ancestral strain. This can be seen in Figure S3, where we plot the estimated densities of evolved and ancestral bacteria at 5.5 hours (soon after cells stopped growing) and at 24 hours. In this experiment 100 µl of a mixture or single clones of overnight ancestral and evolved cells were put in eight flasks each containing 10 mls of LB.



**Figure: S3** Change in the density of ancestral and evolved bacteria in mixed and single clone cultures between 5.5 hours (blue) and 24 hours (maroon). ME and MA are, respectively, the estimated densities of the evolved and ancestral cells in mixed culture. SE and SA are respectively the estimated densities of the evolved and ancestral cells in single clone culture. Means and standard errors of eight cultures of each type.

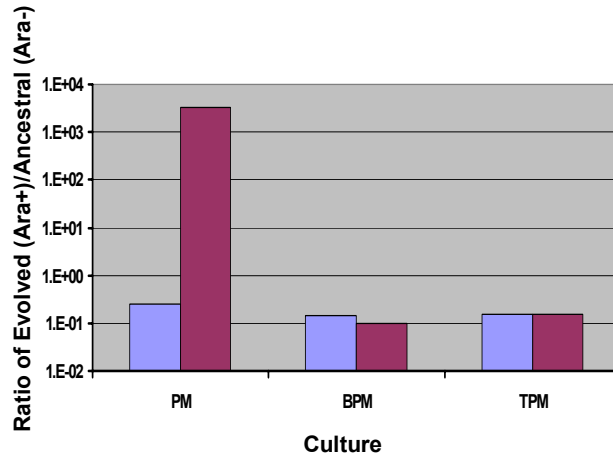
**(ii) Mortality Rates:** The above Figure S3 also illustrates another property of the evolved bacteria. In single clone culture, as determined by CFU data the evolved bacteria die or enter a viable but not culturable state, VBNC, at lower rate than the ancestral. This can be seen from experiments where single clone cultures were sampled soon after they stopped growing and after they had been at stationary phase for at least 15 hours see Figure S4.



**Figure S4: Ratio of later to early stationary phase densities of ancestral and evolved ABM(62-2) in single clone culture; AM and AP, ancestral Ara- and Ara+; EM and EP, evolved Ara- and Ara+. The numbers designate different cultures with different sampling times 1 and 2: 7 and 25 hours; 3: 13 and 23 hours; 4: 5.5 and 24 hours.**

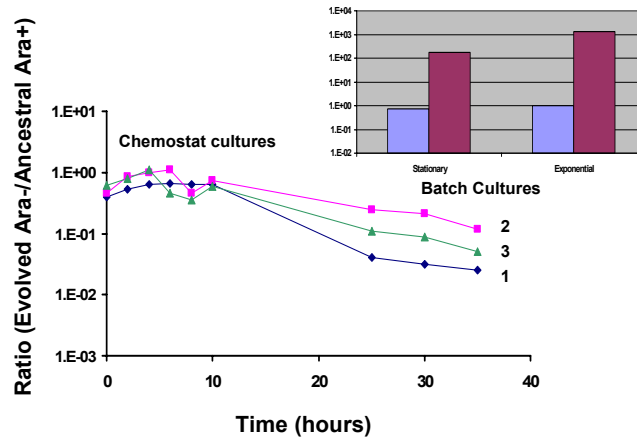
While these separate cultures vary in the extent of “mortality” (reduced number of CFU between time of onset of stationary phases), it is clear than on average the evolved cells die at a lower rate than the ancestral.

**(iii) SCDI requires high pH:** As noted in Figure 1 of our article, the onset of SCDI does not occur until mixed cultures have stopped growing for 12 or so hours. By 24 hours the pH of the unbuffered LB used in these experiments is on the order of 9.0 or higher. To ascertain if this high pH is necessary for SCDI we performed these mixed ancestral-evolved ABM experiments in two buffered versions of LB. In these media, the ingredients of Luria-Bertani broth (per liter 10 mgs Tryptone, 5 mgs yeast extract and 5 grams of NaCl) were added to 60 millimolar solutions of a TRIS or BIS-TRIS. (2-Bis(2-hydroxyethyl)amino-2-(hydroxymethyl)-1,3-propanediol) buffers obtained from Sigma<sup>TM</sup>. The pH’s of these buffered LB solutions were adjusted to 7.0 by the addition of HCl prior to autoclaving. The results obtained in the buffered LBs and their unbuffered control are depicted in Figure S5 . We interpret the absence of SCDI in the pH7.0 buffered medium as consistent with the hypothesis that high pH is a necessary condition for SCDI.



**Figure S5 Ratio of evolved to ancestral ABM at 6 and 24 hours (blue and maroon, respectively) in mixed cultures of unbuffered Luria Bertani broth, PM, and Bis-Tris and Tris buffered LB, BPM and TPM respectively.**

**(iv) SCDI does not occur in continuous culture:** Although it is clear that SCDI occurs when the cells stop growing, the data presented in our article and above do not rule the possibility that SCDI can also occur when the evolved and ancestral bacteria are together for an extended time at high densities but are still replicating. To test this hypothesis we mixed ancestral and evolved cells from chemostats with LB medium. For more information about the design of this “home made” chemostats see the appendix to (Chao et al. 1977) and/or write to us for more details about the more modern versions of these continuous culture devices. To initiate the chemostats overnight cultures of an ancestral, ABM Ara<sup>+</sup> and an evolved (62-2) Ara<sup>-</sup> were put into separate vessels and grown for 72 hours at dilution rates of ~ 0.55 per hour. The vessels were emptied and the evolved and ancestral cells were mixed and 10 mls of this mixture was returned to the vessels of their origin, chemostats 1 and 3. In chemostat 200ul of this mixture was added to LB and allowed to reach full density in the chemostat. As controls we used two batch cultures, one with just the same mixture used in the chemostats without dilution, (stationary), and one where 100 ul of this mixture of evolved and ancestral cells was added to 10 ml LB (exponential). The results of this chemostat experiment are presented in Figure S6.



**Figure S6: Ancestral ABM (62-2) Ara<sup>+</sup> in chemostat and batch culture (insert) with evolved ABM(62-2) Ara<sup>-</sup>, Ratio of evolved to ancestral cells. In the batch culture the blue is the ratio at 5.5 hours and the maroon at 24 hours. In the stationary phase batch culture the mixture of cells used to start the chemostats were mixed without supplemental LB, in the exponential, 100µl of the mixture was added to 10 ml LB.**

We interpret the results of these experiments as support for the hypothesis that SCDI requires the bacteria to be non-growing and presumably in stationary phase. While SCDI occurred in the control stationary phase and initially exponential batch cultures, it did not occur in the chemostat. If anything, in these chemostats the evolved cells had a selective disadvantage relative to the ancestral. There is, however, a caveat to this interpretation, we cannot rule out a pH effect. Although the LB in the above chemostat was not buffered the pH remained between 8.0 and 8.5 which, about 1 pH unit lower than that in the batch control cultures at 24 hour.

### **3 - The genetic basis of SCDI and immunity to SCDI in AB1157Δ*mutS*::*spc* (ABM)**

#### **Plasmid constructions**

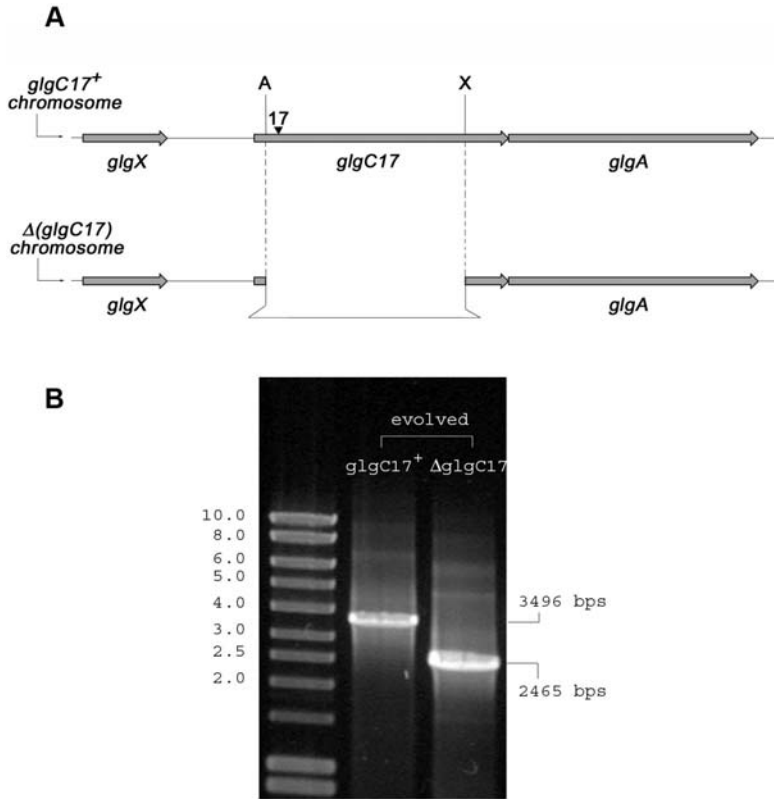
**pMLM02** is a partition-deficient mini-F carrying a chloramphenicol resistance gene (*cat*) that was derived from pMLM1 (Lemonnier et al. 2000) by deleting an *Eco47III*-*HpaI* fragment (3063 bps) containing the *sopABC* operon.

**pCDI1**, that was isolated in this work, derives from the insertion of a 7472 bps fragment (containing the *glgC17*, *glgA*, and *glgP* genes) generated by partial *Sau3A* digestion of the evolved chromosome (see below) into the *BamHI* site of pMLM02.

**pMLM141**, was obtained by inserting a 2158 bps *glgC17*-containing fragment generated by cleavage of pSCDI1 with *XbaI* and *SphI* into a vector obtained by cleaving pBR322 (Balbas et al. 1988) with *NheI* and *SphI*.

**pMLM143** (*cat*, *rpsL*<sup>+</sup>), the integration plasmid designed to knock-out *glgC* in the chromosome, was constructed as follows. A 3496 bps fragment containing the *glgC17* gene and its flanking regions was obtained by PCR amplification using the evolved ABM chromosome as DNA template and the pair of primers *glgC51* (5'-CAGCGACCAGGCATTACTATC-3') and *glgC31* (5'-GTGCTCTCGCAGGTGAAGTTA-3'). The PCR product was digested with *DraI* and *PstI*, followed by end-filling with Klenow fragment of DNA polymerase, prior to being inserted into the *BglII* (end-repaired with Klenow) and *NsiI* sites of the integration-excision vector pLN135 (Cornet et al. 1996) to give pMLM142. The bulk of the *glgC17* gene was then removed by cutting pMLM142 with *AscI* and *XmnI* (Figure S6, A) followed by end-repair using Klenow polymerase and self-ligation of the vector fragment, to give pMLM143.

**Construction of the  $\Delta$ *glgC* strain MLM385.** The multi-step procedure to substitute the  $\Delta$ *glgC17* mutation present in pMLM143 for *glgC17* in the chromosome was performed essentially as described (Lemonnier et al. 2000). Briefly, pMLM143 (*cat*, *rpsL*<sup>+</sup>) was used to transform the streptomycin-resistant (*Sm*<sup>R</sup>) evolved strain. Integration of pMLM143 into the chromosome was selected by plating cells on chloramphenicol-containing medium at 42°C. Excision of *glgC17* from the chromosome was selected by plating on medium containing streptomycin (the excision event being tightly linked to the loss of the pMLM143-borne *rpsL*<sup>+</sup> allele that confers sensitivity to streptomycin). The deletion of *glgC17* in the chromosome was verified by PCR using the oligonucleotides *glgC51* and *glgC31* and by monitoring glycogen accumulation in vivo (Figure 3 in the article) using iodine vapor staining (see below). The  $\Delta$ *glgC17* strain was named MLM385.



**Figure S7. Deletion of *glgC17* in the chromosome of the evolved cells. (A) map of the *glgC* region before and after the knock-out of *glgC17*. The restriction enzymes used to delete a 1032 bps fragment of coding sequences inside *glgC* were *Ascl* (A) and *XmnI* (X). (B) PCR amplification of chromosomal DNA from evolved (left) and evolved  $\Delta$ *glgC17* cells (right) using the oligonucleotides *glgC51* and *glgC31* (see plasmid construction). The size of the expected PCR fragments were 3496 and 2465 bps, respectively. The leftmost lane is a DNA ladder.**

### **Construction of the *E. coli* genomic library and isolation of the *SCDI*<sup>R</sup> clones.**

Genomic DNA was prepared from the evolved ABM strain. After partial digestion by the restriction enzyme *Sau3A*, DNA fragments between 6 and 8 kbp were isolated by agarose gel electrophoresis and ligated into the *Bam*HI site of pMLM02. The random shotgun generated library was electroporated into the ancestral ABM strain using a Bio-Rad MicroPulser™. The electro-transformants were selected at 37°C on LB-agar plates containing chloramphenicol (20  $\mu$ g.ml<sup>-1</sup>). Approximately 4000 transformants were pooled, washed several times in fresh LB medium, diluted to  $\approx 2.5 \times 10^7$  cells/ml and mixed with an equal density of evolved ABM cells. 10 ml of fresh LB medium were inoculated with this mixture and were cultured at 37°C with aeration for 24 hours. Appropriate dilutions were then plated on LB-agar plates containing chloramphenicol.

Approximately 4000 colonies were pooled and were submitted to a new round of competition with evolved cells as described above. The chloramphenicol-resistant colonies obtained after these two rounds were individually tested for resistance to SCDI in pairwise competition experiments with evolved ABM. Clones that showed significant resistance to SCDI were kept for subsequent analyses.

**Assessment of glycogen accumulation by iodine staining.** Staining with iodine vapor (Govons et al. 1969) was used to examine glycogen accumulation in colonies grown for 18 hours at 37°C in Kornberg medium (1.1 % K<sub>2</sub>HP04, 0.85 % KH<sub>2</sub>P04, 0.6 % yeast extract, 1 % glucose).

**Convergent evolution and variation in *glgC* in ABM.** As noted in our article, SCDI evolved in eight separate serial transfer cultures. Clones of the eight evolved strains isolated at the 62<sup>nd</sup> transfer were refractory to SCDI by the other evolved strains and all eight over produced glycogen as measured by the above iodine staining protocol. DNA sequence data of these eight evolved clones indicate that all have missense, base substitutions in *glgC* but at seven different sites. Only two mutations were identical, the proline to serine substitution at the 17<sup>th</sup> codon (Table 1). In other words, the evolution of SCDI in ABM was convergent in the sense that the same phenotype evolved through different mutations.

**Table 1 : *glgC* sequence variation in the evolved ABM strains from the 62<sup>nd</sup> transfer**

Evolved strain	Codon change	Site and amino-acid change
62.1	CGC /CAC	R14H
62.2	CCA/TCA	P17S
62.3	GGC/GAC	G336D
62.4	ATC/TTC	I66F
62.5	GAC/TAC	D125Y
62.6	CTT/ATT	L330I
62.7	CCA/TCA	P17S
62.8	TTC/CTC	F318L

#### 4- Glycogen production is not needed for immunity to SCDI but is needed for inhibition

In an effort to ascertain the role of glycogen production in this contact-dependent inhibition using AB1157 *mutS*<sup>+</sup> we constructed three strains with different combinations of *glg* alleles and tested their sensitivity to SCDI by evolved 62-2 and ability to inhibit the ancestral ABM in mixed culture experiments. The strains constructed for these tests were:

**An** – AB1157 *glgC17* (a replacement of the *glgC* gene with the evolved *glgC17* from 62-2, ABM *glgC17*)

**glgA** - AB1157 *glgC17 glgA* (**An** with a knock out of *glgA*, glycogen synthase)

**glgP** – AB1157 *glgC17Δ glgP* (**An** with a knock out of *glgP*, glycogen phosphorylase )

The bacteria and methods employed for these constructions are described below.

#### Procedures for constructing An and the *glgA* and *glgP* knockouts.

**Bacteria, bacteriophage, and growth conditions.** Bacterial strains and bacteriophage are described in **Table 2**. Unless noted, bacterial strains were grown at 37 °C in Luria-Bertani (LB) medium (Miller 1972) or Kornberg medium (KB) (1.1 % K<sub>2</sub>HP04, 0.85 % KH<sub>2</sub>P04, 0.6 % yeast extract, 1 % glucose). Media were solidified using 1.5% agar and were supplemented with antibiotics, as needed, at the following concentrations: ampicillin 100 µg ml<sup>-1</sup>; chloramphenicol 25 µg ml<sup>-1</sup>; and tetracycline 10 µg ml<sup>-1</sup>.

**Table 2. Bacterial strains, plasmids, phage and growth conditions.**

Strain, plasmid or phage	Description or genotype	Source or reference
<i>E. coli</i> K12 Strains		
MG1655	F <sup>-</sup> λ <sup>-</sup>	Michael Cashel
CAG18450	MG1655 <i>zhf-50::Tn10</i>	Singer, et.al., 1989(Singer et al. 1989)
ABM62-2MS	AB1157 <i>glgC17 mutS</i>	This study
MG1655-GA12	MG1655 Δ <i>glgA</i>	X. Wang, unpublished
ABM18450	AB1157 <i>zhf-50::Tn10</i>	This study
AB1157	Standard Lab strain	E. A. Adelberg (CGSC)

ABM62-2MS18450	AB1157 <i>glgC17 mutS zhf-50::Tn10</i>	This study
AB18450C17-1 Designated <b>An</b>	AB1157 <i>glgC17 zhf-50::Tn10</i>	This study
AB18450C17GA1 Designated <b>glgA</b>	AB1157 <i>glgC17 zhf-50::Tn10 ΔglgA::cat</i>	This study
AB18450C17GP1 Designated <b>glgP</b>	AB1157 <i>glgC17 zhf-50::Tn10 ΔglgP::cat</i>	This study
Phage		
P1 <i>vir</i>	Strictly lytic P1	Carol Gross
Plasmids		
pKD46	For arabinose induction of λ Red system	Datsenko and Wanner, 2000(Datsenko & Wanner 2000)
pKD3	Contains the <i>cat</i> gene	Datsenko and Wanner, 2000(Datsenko & Wanner 2000)

**Strain construction.** Strains AB18450C17GA1 ( $\Delta glgA$ ) and AB18450C17GP1 ( $\Delta glgP$ ) were constructed as follows. First, P1*vir* was used to transduce the tetracycline resistance (*tet*) gene from mapping strain CAG18450 (*zhf-50::Tn10*) (Singer et al. 1989) to strain AB62-2MS carrying the *glgC17* allele. A transductant exhibiting both tetracycline resistance (Tet<sup>R</sup>) and high glycogen accumulation, as determined by dark colony staining upon exposure to iodine vapor when grown overnight on KB agar plates, was isolated and designated AB62-2MS18450. The *glgC17* allele and *zhf-50::Tn10* were then co-transduction to a *mutS* wild type strain of AB1157 to create strain AB18450-C17-1, by selecting for Tet<sup>R</sup> and screening for high glycogen accumulation. The *glgA* and *glgP* genes of AB18450C17-1 and the *glgA* gene of MG1655 were replaced with a chloramphenicol (*cat*) expressing cassette by λ-Red mediated gene replacement (Datsenko & Wanner 2000) to create strains AB18450C17GA1, AB18450C17GP1 and MG1655GA12, respectively. The *cat* cassettes used to replace *glgA* and *glgP* were PCR-amplified from the template plasmid pKD3 using primer sets (for *glgA*) AH1P1 (5'-aatgctacggaagtagggcataaacaggagcgcgataagtgtaggctggagctgcttc), AH2P2 (5'-

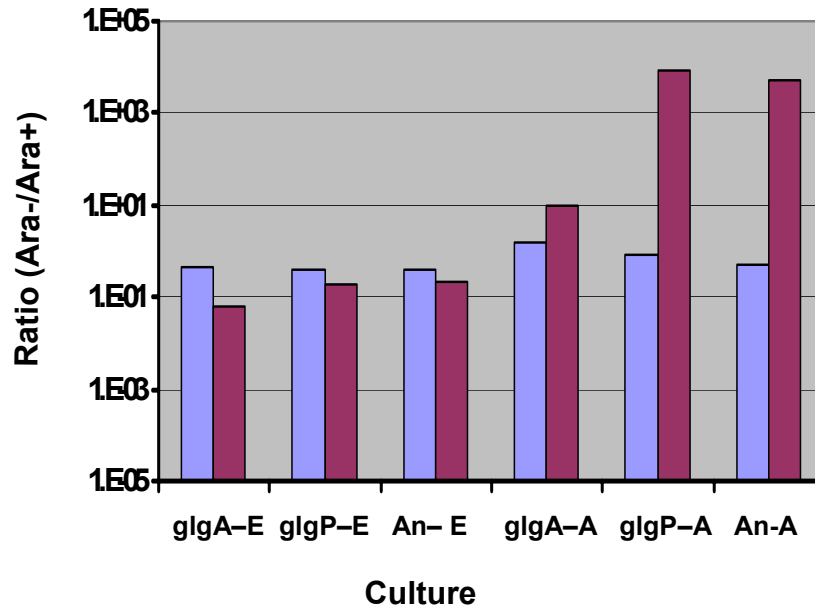
gctaagcgtgggcgatgaatatgtaaaccggagcattcatatgaatatacctccttagt) and (for *glgP*) PH1P1 (5'-gcaagtcgtaccgtgagctttactatcgcttgaaataggtgtaggctggagctgcttc), PH2P2 (5'-gatcgatatgccagatatgatcggcgtactctttgatagtcatatgaatatacctccttagt). Conditions for amplification of the *cat* cassette from pKD3 were as follows: 1 cycle at 94°C for 1 min.; 30 cycles of 94°C for 30 seconds, 65°C for 30 sec., 68°C for 90 sec.; 1 cycle at 68°C for 10 min. The PCR product containing the *cat* gene with terminal *glgA* or *glgP* homology regions, was treated with DpnI, gel purified and introduced by electroporation into MG1655[pKD46] and AB18450C17-1[pKD46], which had been grown in LB containing arabinose (1 mM). Transformants were selected for chloramphenicol resistance (Cam<sup>R</sup>) and screened for loss of glycogen accumulation. The targeted gene replacements were confirmed by PCR using the primers (for *glgA*) Aconfirm1 (5'-tagccacgggatgacccttaactc), Aconfirm2 (5'-tgggcacggttgaacgtaacc) and (for *glgP*) Pconfirm1 (5'-ttactgtggcggtttgtgc), Pconfirm2 (5'-ggatgccgaaaaagtcattac). Confirmatory PCR conditions were as follows: 1 cycle at 94 °C for 1 min.; 30 cycles of 94 °C for 1 min., 53 °C for 1 min., 68 °C for 3 min.; 1 cycle at 68 °C for 10 min. A non-polar deletion of the *cat* cassette from strain MG1655-GA12 was constructed using FLP-mediated recombination at flanking FRT sites (Datsenko & Wanner 2000). This deletion was confirmed by PCR as described above. We thank Xin Wang who created MG1655-GA12 and designed the primers for amplification and confirmation of the pKD3 *cat* cassette replacement of *glgA* and *glgP*.

### **Mixed culture SCDI experiments with these knockouts**

The above described An, *glgA* and *glgP* Ara<sup>-</sup> strains were mixed with evolved ABM Ara<sup>+</sup> (62-2) and with the ancestral ABM Ara<sup>+</sup> and put into LB 100 ul to 10 ml in 50ml flasks. The densities of these competing strains were estimated at 5 hours (when cell growth ceased) and 24 hours. The results of this experiment are presented in Figure S10.

The *glgA* construct (AB1157 *glgA glgC17*) did not produce glycogen and was not inhibited by the evolved ABM *glgC17*. On the other hand when this strain was mixed with the ancestral ABM it did not cause SCDI. We interpret these results to indicate that glycogen production is not essential for the immunity of AB1157 to SCDI by the evolved *glgC* strains but is required for the inhibition of ancestral cells. On the other

hand, the glycogen producing AB1157 *glgC17* and AB1157 *glgC17 glgP* constructs did inhibit the



**Figure S8** The constructed *glg* variants of AB1157 *glgC17* Ara- (*glgA*, *glgP* and *An*) in mixed cultures with the evolved Ara+ (E) and ancestral Ara+ (A) ABM. Ratio of the Ara- constructs with the Ara+ ancestral and evolved ABM strains at 5 hours (blue) and 24 hours (maroon).

ancestral strain. While this suggests that glycogen catabolism is not required for SCDI in ABM cells, other minor enzymatic systems can also support glycogen turnover which maybe sufficient to promote inhibition.

### 5-Cell viability analysis during SCDI using confocal microscopy

The SCDI phenomenon described here is based on the results of plating experiments the relative abilities of the mixed strains to form colonies on agar. Not clear from these result is whether the failure of the ancestral cells to form colonies in mixed culture with the evolved cells is due to contact-mediated killing or contact with these inhibiting bacteria inducing a viable but non-culturable (VBNC) state.

To begin to address this question we performed a confocal microscopy analysis of mixed cultures of evolved and ancestral ABM cells after 28 hours of co-incubation at 37°C. As can be seen in Figure S9 panel a, ancestral cells expressing the DsRed protein were present at similar proportions and displayed similar fluorescence intensities irrespective of whether they were in mixed cultures with GFP-labelled evolved cells or mixed with GFP-labelled ancestral cells. In the former case the plating efficiency (CFU) of ancestor cells was less than  $10^{-3}$  that of the evolved, as expected (data not shown). These observations were fully consistent with the results from the FACS experiment where no significant variation in the fluorescence of ancestral cells expressing the DsRed protein was observed, despite a drop of several orders of magnitude in their plating efficiency when mixed with evolved cells during 28 hours (Figure 3). These experiments suggested that the ancestral cells remained viable during SCDI in stationary phase cultures. To further address this hypothesis we performed a propidium iodide (PI) inclusion assay, which is used as a standard criterion to distinguish dead from viable bacterial cells. Bacteria that undergo membrane disruption and cell death appear as red fluorescing cells following PI inclusion (Boulos et al. 1999). Our results indicated that death was not occurring in bacterial cultures experiencing SCDI. As shown in Figure S11, panel b, no PI inclusion could be detected in cells from either the ancestors/evolved or the ancestors(GFP)/ancestors mixed cultures. At the same time the cells were visualized by staining with 4',6-diamidino-2-phenylindole (DAPI), which yields blue fluorescing cells upon binding to DNA. In addition, green fluorescence was clearly detected in GFP-producing ancestral cells. When the mixed cultures were exposed to polymyxin B (an antimicrobial compound that binds to cell membranes and leads to rapid cell disruption (Lehtinen et al. 2006)) red fluorescence due to PI inclusion was clearly observed.

Altogether, our results support the hypothesis that ancestral cells enter a viable but not culturable (VBNC) state upon contact-dependent inhibition in stationary-phase cultures. However, at this stage we cannot predict whether this VBNC state remains unaltered or rather evolves to rapid cell death upon dilution of the cultures in fresh medium and plating on agar. Indeed, this is a matter of ongoing debate in the field of VBNC research (Nystrom 2003; Oliver 2005)). In this respect, SCDI in stationary phase has inherent properties (like culturable (evolved) and non-culturable (ancestors) cells co-existing and being easily sorted and purified; good knowledge of the parameters that control the phenomenon (cell frequency and density,

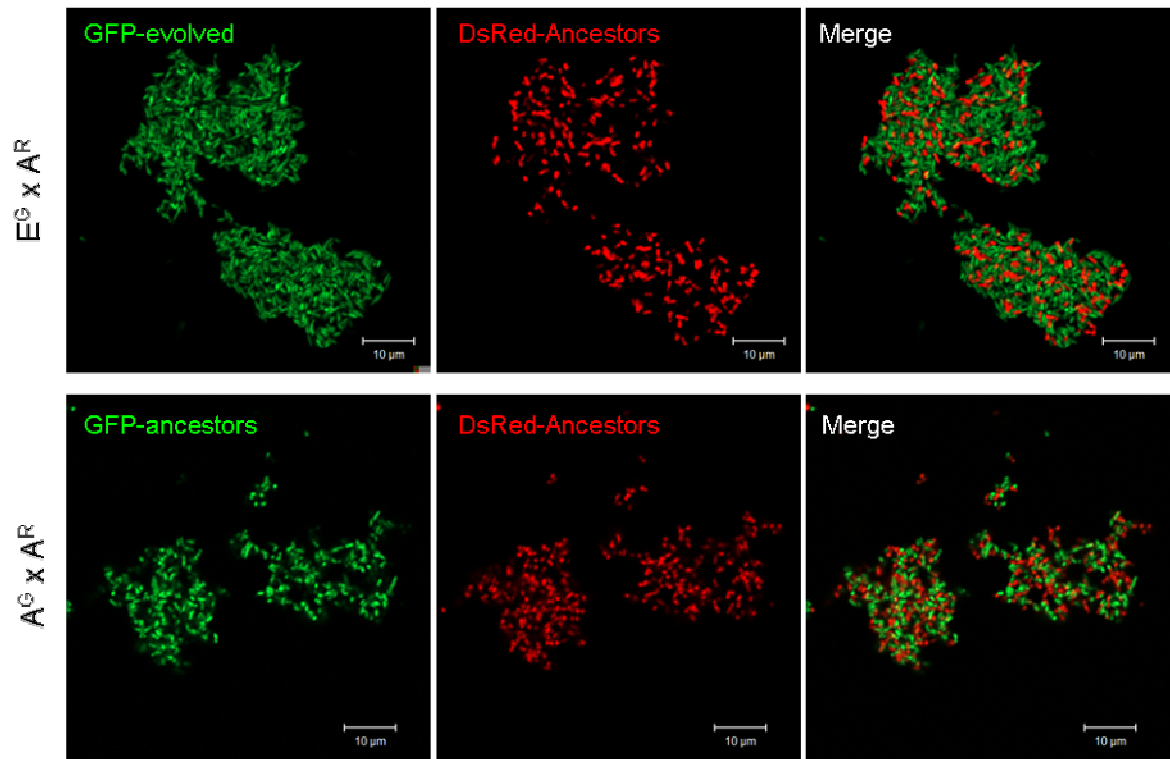
pH) that we anticipate will make it a prominent model for VBNC studies. Particularly, it will be interesting to determine whether cell deterioration via protein carbonylation is specifically taking place in the ancestral cells during SCDI, as reported in the case of non-culturable cells in starving *E. coli* populations (Desnues et al. 2003).

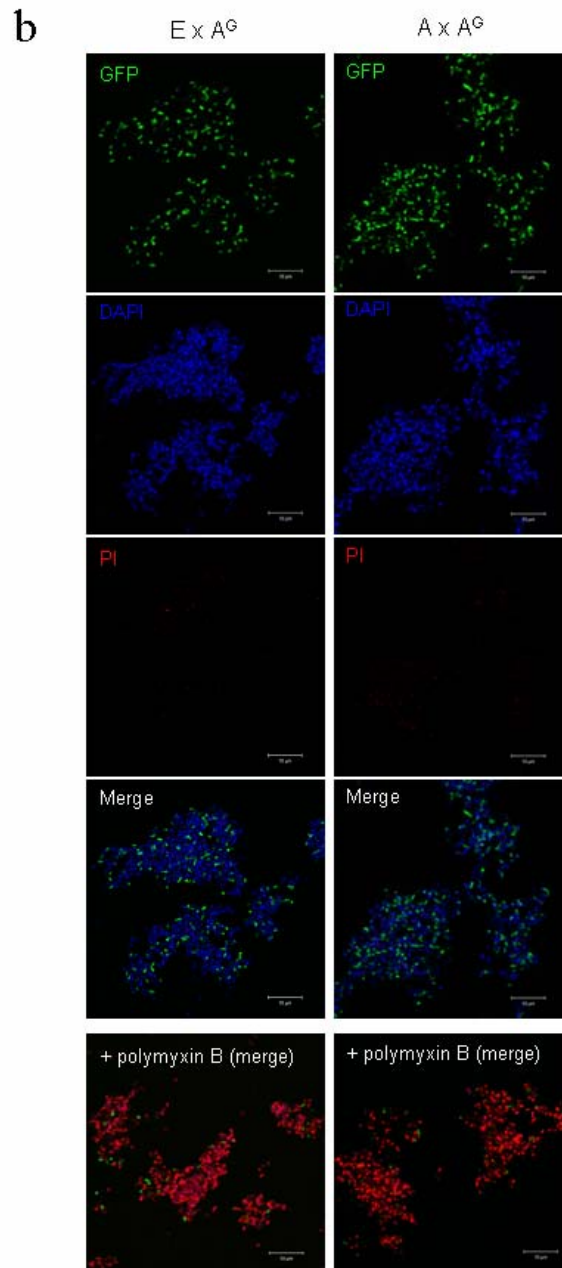
### **Experimental procedures for the Confocal Microscopy**

Confocal laser scanning microscopy was performed using a Zeiss 510 META Confocal instrument. Excitation wavelengths used to elicit red, blue or green fluorescence were 543, 405 and 488 nm, respectively. DsRed- and GFP-labelled *E. coli* cells were prepared as described in the general experimental procedures. For bacterial cell viability analyses, aliquots (500  $\mu$ l) of 28 hours mixed cultures of evolved and ancestral ABM were centrifuged and the pellets were resuspended in PBS (10 ml). 1 ml aliquots of this resuspension were treated with propidium iodide (PI, 2.5  $\mu$ g. ml<sup>-1</sup>) and DAPI (4',6-diamidino-2-phenylindole, 2  $\mu$ g. ml<sup>-1</sup>). The samples were kept for 10 min at room temperature in the dark. The stained suspensions were then centrifuged and the pellets were resuspended in Moviol (10  $\mu$ l) and mounted in coverslips. The samples were observed and photographed using confocal microscopy.

Exposure of the bacterial cultures to polymyxin B was performed by adding 1.5 ml fresh LB supplemented with polymyxin B (5  $\mu$ g. ml<sup>-1</sup>) to aliquots (500  $\mu$ l) of the same 28 hours mixed cultures used above. The samples were incubated at 37°C for 15 min and then were centrifuged and resuspended in PBS (10 ml) prior to being treated with IP and DAPI as above.

**a**

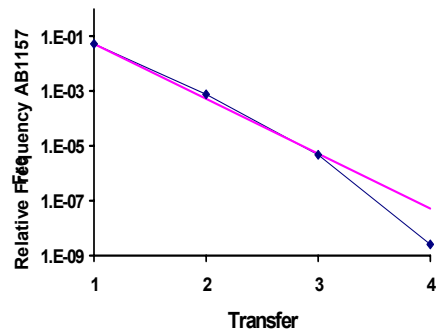




**Figure S9. Cell viability analysis of SCDI using confocal microscopy. a) Fluorescent emission of DsRed-labelled ancestral ABM (A<sup>R</sup>) mixed with either GFP-labelled evolved (E<sup>G</sup>) or GFP-labelled ancestral cells (A<sup>G</sup>) at a 1:1 initial ratio and grown together for 28 hours at 37°C. b) The integrity of bacteria in 28 hours mixture of GFP-labelled ancestral cells (A<sup>G</sup>) with unlabelled evolved (E) or ancestral (A) cells was monitored using propidium iodide (PI). In addition, bacterial cells were visualized through staining of their nucleoids by using DAPI. The lower micrographs show the cell samples after treatment with the membrane damaging compound polymyxin B**

### SCDI is not due to plating efficiency differences:

If, as the preceding results suggest, SCDI does not operate through killing but rather via the induction of a viable but not culturable state; it is conceivable that this VBNC state is only manifest by the inability of these bacteria to form colonies on agar. Were they to remain in liquid LB, they would grow again. To ascertain whether this is the case, we did serial passage experiments with AB1157 and MG1655. These strains, rather than the evolved and ancestral ABM were used for this experiment for two reasons. First AB1157 had a marker *rpsL* (Str-r) that enable us, by selective plating, to measure its frequency when rare in cultures with MG1655 (Str-s), which was not the case for the ABM evolved and ancestral strains. Second, because of the high mutation rate in ABM rare mutants for a single allele are commonly produced by mutation from the dominant strains. In these experiment 100  $\mu$ l of a mixture of MG1655 and AB1157 were introduced into 10 ml LB in 50 ml Erlenmeyer flasks. Samples were taken each day and 100  $\mu$ l of the mixture was transferred to a fresh flask with 10 ml of LB. The densities of these two cell lines were estimated on Tetrazolium arabinose agar and/or streptomycin LB agar to detect AB1157 (*rpsL*) when it was rare. The results of one of these experiments are presented in Fig.S12. The “Expected line is that which would obtain if AB1157 declined by a factor of 100 (the dilution factor) at each transfer.



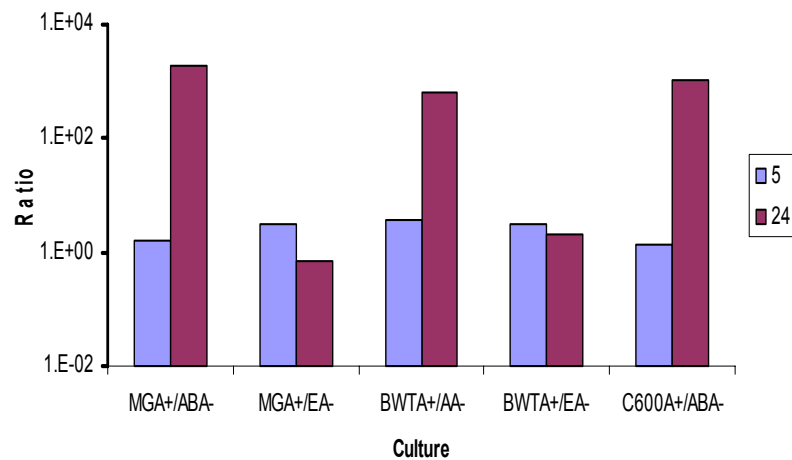
**Figure S10** Observed (blue) and Expected (fusia) changes in the relative frequency of AB1157 in successive 1:100 serial transfers in mixed LB culture with MG1655. The expected decline in the relative frequency of AB1157 was calculated under the assumption of 100 fold reduction in the density of AB1157 at each transfer.

If the inhibition was due to contact with MG1655 solely reducing the ability of AB1157 to form colonies, the relative frequency of AB1157 would not continue to decline in successive passages in liquid culture as it does.

## 6- Evidence for SCDI with other strains of *E. coli*

### *E. coli* K-12 MG1655, C600 and wild-type *E. coli* B as inhibitors of

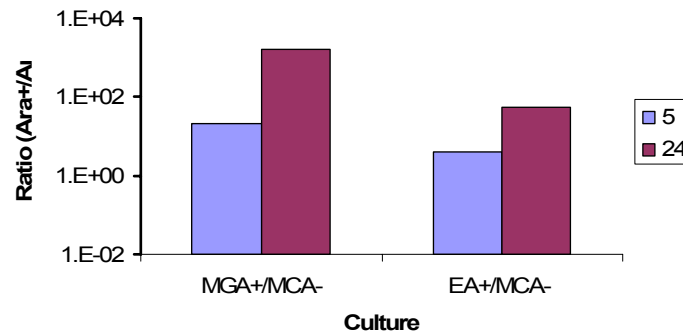
**AB1157 $\Delta$ mutS::spc nal (ABM) and AB1157mutS<sup>+</sup>** : Overnight Ara<sup>+</sup> cultures of *E. coli* K-12 MG1655 (MGA<sup>+</sup>), C600 Ara<sup>+</sup> (C600A<sup>+</sup>) and a wild-type strain of *E. coli* B (BWTA<sup>+</sup>), were mixed with Ara<sup>-</sup> AB1157mutS<sup>+</sup> (ABA<sup>-</sup>), an Ara<sup>-</sup> ancestral ABM (AA<sup>-</sup>) or an evolved variant of this strain, EA<sup>-</sup>. For each mixed pair of overnight cultures 100  $\mu$ l put into 10 ml LB and were incubated with shaking at 37<sup>o</sup> C. Samples were taken at 5 hours, when the cultures were no longer growing, and again at 24 hours and the estimated densities of Ara<sup>+</sup> and Ara<sup>-</sup> cells estimated from CFU data. The results of one of these experiments are depicted in Figure S11.



**Figure S11: SCDI with other strains; ratio of Ara<sup>+</sup> to Ara<sup>-</sup> in mixed cultures at 5 and 24 hours. See the text for the strain designations.**

We interpret these results as evidence that when cultured with either *E. coli* K-12 MG1655, C600 or wild-type *E. coli* B, the *E. coli* K-12 strains AB1157*mutS*<sup>+</sup> or ancestral ABM employed in the evolution experiments are subject to inhibition at stationary phase. These results also indicate that the evolved *glgC17* mutant of ABM is immune to inhibition by either *E. coli* K-12 MG1655 or *E. coli* B. Since there was no evidence for the stationary phase AB1157 dying at higher rates in sterile filtrates of their own cultures than in filtrates of stationary phase cultures of MG1655 or *E. coli* B we conclude that this inhibition requires cell-cell contact, i.e. is SCDI (data not shown).

**MC1061 is subject to SCDI:** Overnight LB cultures of *E. coli* K-12 strain MC1061 (Ara<sup>-</sup>) (MCA<sup>-</sup>) were mixed with MG1655 (MGA<sup>+</sup>) or the evolved ABM Ara<sup>+</sup> (EA<sup>+</sup>) and 100µl of the mixtures were put into 10 ml LB. Samples were taken at 5 hours, when the cultures were no longer growing, and again at 24 hours. The results of one of these experiments is depicted in Figure S12



**Figure S12: MC1061 is subject to SCDI: Changes in ratio of Ara<sup>+</sup> and Ara<sup>-</sup> between 5 and 24 hours; MG1655 (MGA<sup>+</sup>), or an Ara<sup>+</sup> evolved ABM with MC1061 (MCA<sup>-</sup>)**

We interpret the results of this experiment as support for the hypothesis that *E. coli* K-12 strain MC1061 is sensitive to inhibition at stationary phase when mixed with either *E. coli* MG1655 or the evolved ABM. The extent of the inhibition is somewhat less when it is mixed with the evolved ABM strain than with MG1655. Filtrate experiments similar

to those considered above, support the interpretation that this inhibition of MC1061 requires physical contact with these other bacteria, i.e. is contact dependent, SCDI (data not shown).

## **7 - Population and Evolutionary Dynamics of SCDI: Theoretical Considerations:**

**(i) The frequency- and density- dependence of SCDI is anticipated from a simple mathematical model.** To model contact-dependent inhibition we consider a non-growing population of evolved and ancestral cells with densities  $E$  and  $A$  bacteria per ml, respectively. We assume: (i) the  $E$  and  $A$  bacteria and die at rates  $d_e$  and  $d_a$   $h^{-1}$  respectively due to processes that have nothing to do with inhibition by a competing population, (ii) bacteria in these cultures randomly collide with each other at rates proportional to the product of their densities; a fraction of contacts with the evolved strain, the parameter  $\tau$ , prevents the ancestral strain from replicating (kills them or induces a VBNC state). It should be noted, that this model of SCDI does not assume that the inhibition occurs through a single contact by pairs of individual cells or that cells remain in permanent contact, it is a mass process that may require many contacts by groups of cells. It also does not assume that the inhibited cells are dead, but rather that they no longer replicate or can be detected by forming colonies, i.e. they can be viable but not culturable, VBNC. With these definitions and assumptions, the rates of change in the densities of the inhibited (dying) populations of ancestral and evolved cells are given by,

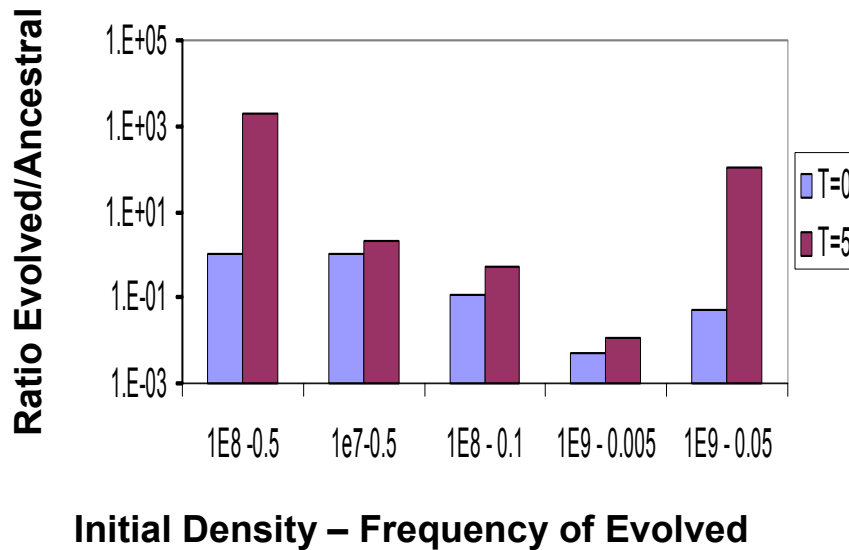
$$dA/dt = -d_a A - \tau A E$$

$$dE/dt = -d_e E$$

To illustrate the properties of this model, we use a numerical solution to these equations (a computer simulation). This and the other simulations used here were programmed in Berkeley Madonna<sup>TM</sup>. Copies of this program, which is the same program used for the evolution simulations below can be downloaded from [www.eclf.net](http://www.eclf.net).

As can be seen in the below simulation (Figure S13), when the total density of bacteria ( $A+E$ ) or the frequency of the inhibitor strain ( $E$ ) are low SCDI has little effect on the

number of ancestral cells recovered. It should be noted, that frequency- and density-dependence would also be obtained if the inhibition of the ancestral cells was mediated by an extracellular toxin like a bacteriocin (Levin 1988).



**Figure S13 Simulation results: Changes in ratio of Evolved and Ancestral cells at different initial total cell densities and different initial frequencies of evolved bacteria (e.g. 1E8- 1.0) is an initial cell density of  $1 \times 10^8$  and an initial relative frequency of the evolved strain of 0.5. The parameter values in these runs,  $d_a=d_e=0.02$  per hour and  $\tau = 10^{-11}$  were chosen to illustrate the properties of this model and are not derived from actual estimates.**

**(ii) Mathematical model and computer simulation of the evolution of SCDI**

In this model we assume that within a transfer there are two phases. In Phase 1 there is no contact-dependent inhibition of the ancestral cells. The rates of change in the densities of the ancestral and evolved populations, A and E, respectively, are solely a function of their resource concentration dependent growth rates and a constant death rate. For the former we use a Monod function (Monod 1949),  $v_a\Psi(R)$  and  $v_e\Psi(R)$ , where  $\Psi(R)=R/(R+k)$ , where R is the concentration of the limiting resource,  $v_a$  and  $v_e$  are the maximum hourly growth rates of the ancestral and evolved cells respectively and  $k \mu\text{g}$  is the concentration of the resource where the growth rate is half of its maximum value, the Monod constant. As in (Stewart & Levin 1973) we assume, the resource is taken up at

rates proportional to the densities of the bacterial populations, their replication rates (the Monod functions) and a parameter,  $e$ , the conversion efficiency; the production of a new cell requires  $e \mu\text{g}$  of resource. With these definitions and assumption, the rates of change in resource concentration and density of bacteria during Phase 1 is

$$dR/dt = -\Psi(R) [A*v_a + E*v_e]e$$

$$dA/dt = A*v_a*\Psi(R) - d_a*A$$

$$dE/dt = E*v_e*\Psi(R) - d_e*E$$

where  $d_a$  and  $d_e$  are, respectively, the death rates for the ancestral and evolved bacteria.

When the time within a transfer exceeds a defined period, 16 hours in our simulations, Phase 2 commences. Phase 2 is the situation described above the populations are no longer growing but continue to “die” at the density independent rates,  $d_a$  and  $d_e$ , and the ancestral cells die (or enter a VBNC state) at a rate proportional to the product of the densities of the ancestral and evolved cells and an SCDI rate constant,  $\tau \text{ ml} \times \text{cell per hour}$ . With these assumptions and definitions the rates of change in the density of the bacteria in Phase 2 are given by,

$$dA/dt = -d_a * A - \tau * A * E$$

$$dE/dt = -d_e * E$$

In these simulations the population is maintained by serial passage with a transfer every 24 hours. At each transfer, cells surviving the previous cycle are diluted by a factor of 0.01, and  $R$  is set equal to 1000 and the growth cycle starts again. In the runs made in Figure 5, the population was initially composed of  $10^7$  ancestral cells and no evolved bacteria. The evolved bacteria arose by recurrent mutation at rate  $\mu$  per cell per generation from the ancestral population which we simulated by a Monte Carlo process. At each finite time interval  $\Delta t$  a pseudo random number  $x$  ( $0 \leq x \leq 1$ ) from a rectangular distribution. When  $x < N * \Delta t * \Psi(R) * \mu$ , a single mutant cell was added to the  $E$  population. We did not consider recurrent mutation to the  $A$  state from  $E$ . The parameter values used for the runs made in Figure 5 in our article are presented in Table 2.

Table 3 – Parameter values used for the simulations presented in Figure 5\*

Parameter	Definition	A	B	C
va	Maximum growth rate A	1.0	1.0	1.0
ve	Maximum growth rate E	<b><u>0.99</u></b>	1.0	1.0
e	Conversion efficiency	$5 \times 10^{-7}$	$5 \times 10^{-7}$	$5 \times 10^{-7}$
k	Monod Constant	0.25	0.25	0.25
da	Death rate A	0.02	0.02	<b><u>0.025</u></b>
de	Death rate E	0.02	0.02	0.02
$\tau$	SCDI killing constant	$10^{-11}$	$10^{-11}$	$10^{-11}$

\* These parameter values were chosen to illustrate the process rather than estimated experimentally, although the population growth and resource uptake and use parameters are in a realistic range.

## REFERENCES

- Balbas, P., Soberon, X., Bolivar, F. & Rodriguez, R. L. 1988 The plasmid, pBR322. *Biotechnology* 10, 5-41.
- Boulos, L., Prevost, M., Barbeau, B., Coallier, J. & Desjardins, R. 1999 LIVE/DEAD BacLight : application of a new rapid staining method for direct enumeration of viable and total bacteria in drinking water. *J Microbiol Methods* 37, 77-86.
- Chao, L., Levin, B. R. & Stewart, F. M. 1977 A complex community in a simple habitat: an experimental study with bacteria and phage. *Ecology* 58, 369-378.
- Cornet, F., Louarn, J., Patte, J. & Louarn, J. M. 1996 Restriction of the activity of the recombination site dif to a small zone of the Escherichia coli chromosome. *Genes Dev* 10, 1152-61.
- Datsenko, K. A. & Wanner, B. L. 2000 One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. *Proc Natl Acad Sci U S A* 97, 6640-5.
- Desnues, B., Cuny, C., Gregori, G., Dukan, S., Aguilaniu, H. & Nystrom, T. 2003 Differential oxidative damage and expression of stress defence regulons in culturable and non-culturable Escherichia coli cells. *EMBO Rep* 4, 400-4.

- Govons, S., Vinopal, R., Ingraham, J. & Preiss, J. 1969 Isolation of mutants of Escherichia coli B altered in their ability to synthesize glycogen. *J Bacteriol* 97, 970-2.**
- Lehtinen, J., Jarvinen, S., Virta, M. & Lilius, E. M. 2006 Real-time monitoring of antimicrobial activity with the multiparameter microplate assay. *J Microbiol Methods* 66, 381-9.**
- Lemonnier, M., Bouet, J. Y., Libante, V. & Lane, D. 2000 Disruption of the F plasmid partition complex in vivo by partition protein SopA. *Mol Microbiol* 38, 493-505.**
- Levin, B. R. 1988 Frequency-dependent selection in bacterial populations. *Philosophical Transactions of the Royal Society of London - Series B: Biological Sciences* 319, 459-72.**
- Miller, J. H. 1972 *Experiments in Molecular Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Press.**
- Monod, J. 1949 The growth of bacterial cultures. *Annual Review of Microbiology* 3, 371-394.**
- Nystrom, T. 2003 Conditional senescence in bacteria: death of the immortals. *Mol Microbiol* 48, 17-23.**
- Oliver, J. D. 2005 The viable but nonculturable state in bacteria. *J Microbiol* 43 Spec No, 93-100.**
- Singer, M., Baker, T. A., Schnitzler, G., Deischel, S. M., Goel, M., Dove, W., Jaacks, K. J., Grossman, A. D., Erickson, J. W. & Gross, C. A. 1989 A collection of strains containing genetically linked alternating antibiotic resistance elements for genetic mapping of Escherichia coli. *Microbiol Rev* 53, 1-24.**
- Stewart, F. M. & Levin, B. R. 1973 Resource partitioning and the outcome of interspecific competition: a model and some general considerations. *American Naturalist* 107, 171-198.**