

ENVIRONMENTAL VISCOSITY DOES NOT AFFECT THE EVOLUTION OF COOPERATION DURING EXPERIMENTAL EVOLUTION OF COLICIGENIC BACTERIA

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Cooperation should be favored under environmental conditions allowing the preferential interaction of cooperators among themselves and limiting interactions with defectors. Bacteria cooperating to kill competitors by secreting a toxin evolved during several hundred generations in two environments: a viscous environment that should promote cooperator assortment, and a nonviscous environment that should not allow such preferential interaction. A quantitative decrease in cooperation was observed in all populations, but as expected, cooperation was maintained at a higher level in the viscous environment. Mutants that are resistant against but not producing the toxin were identified at a low frequency in a few populations from the viscous environment and at a high frequency in all the populations from the nonviscous environment. The underlying mutations were identified. Relative fitness of the cooperator and mutant genotypes were obtained with bacteria that were isogenic, except for the identified mutations. Competition experiments indicated that cooperation is not favored by environmental viscosity as imposed in our system and suggested that when it comes to cooperation, environmental viscosity should be considered not only in terms of individual movement, but also in terms of the distribution of the public good.

KEY WORDS: Altruism, cooperator, defector, experimental evolution, interference competition.

Altruistic acts producing benefits for others at the expense of future reproduction of the altruistic individual are central to the functioning of many biological communities. The conditions required for the emergence and persistence of such behavior despite the possibility of exploitation by nonaltruists have been extensively studied, but the basic conceptual requirement for the evolutionary maintenance of cooperation is easy to understand: altruistic individuals must interact preferentially among themselves and avoid interaction with selfish individuals (Fletcher and Doebeli 2009).

One of the simplest scenarios allowing preferential interaction among cooperators occurs when evolution occurs in viscous, spatially structured environments. This study aims at comparing the evolution of cooperation in viscous and nonviscous environments using colicigenic bacteria as a model system. Colicins are

toxins produced by the bacteria *Escherichia coli*. These toxins are part of the bacteriocins, a family of antimicrobial proteins probably playing an important role for avoiding competition in microbial communities (Kerr et al. 2002; Riley and Wertz 2002). They are characterized by a narrow killing spectrum, are lethal to bacteria closely related to the producing strain, and are found in almost every bacterial species examined (Riley and Wertz 2002). Colicin production has a fairly simple genetic basis. It is encoded on an operon composed by an SOS promoter followed by a colicin gene, which encodes the toxin; an immunity gene, which encodes a protein conferring a specific immunity to the producer cell; and a lysis gene, encoding for a protein involved in colicin release through lysis of the producer cell (Cascales et al. 2007). This operon is regulated by the bacterial SOS system,

an inducible DNA repair system that allows bacteria to survive sudden increases in DNA damage (Radman 1974). Colicin expression is repressed by the binding of LexA to a site called SOS boxes located upstream of the colicin operon. The activation of the SOS system leads to the autocleavage of LexA, freeing the SOS boxes and allowing the initiation of the transcription of the colicin operon. Moreover, the immunity gene is under the control of its own constitutively active promoter, allowing the constitutive immunity of the cells carrying the colicogenic plasmids. In normal conditions, a population of isogenic colicogenic bacteria consists of a small proportion of producing cells and a large proportion of nonproducing cell (the proportion of producing cells has been estimated to be 0.1% for colicin E2, Cascales et al. 2007). In conditions of stress, the proportion of producing cells tends to increase (Cascales et al. 2007).

During the last 10 years, the notion of cooperation has started to be applied to microorganisms (Crespi 2001; Velicer 2003; Travisano and Velicer 2004; West et al. 2007), and colicogenic bacteria have been used in several studies relevant to the study of the evolution of cooperation (Chao and Levin 1981; Kerr et al. 2002; Kirkup and Riley 2004). Colicin can be considered as a common good, which by interference competition benefits the population of colicogenic bacteria, allowing the allocation of more resource to bacteria resistant to the toxin, and coming at the cost of death of the producing cells. In the present study, populations of colicogenic bacteria were allowed to evolve during several hundred generations in the presence of sensitive competitors in either a very viscous (the surface of an agar plate) or a nonviscous (a homogenized liquid medium) environment.

The evolution of colicin production was expected to be different in the two evolution environments. In the nonviscous environment, no matter how much common good is present in the environment, its benefits are equally distributed among all the members of a given population. As genotypes producing less colicin pay a lesser cost, genotypes producing less or even no colicin but are still immune to the colicin (i.e., defectors) are going to be favored by selection and should drive the cooperators to extinction. In the viscous environment, genotypes producing less or no colicin could also start invading the population by taking advantage of the colicin without paying the cost of its production, but by doing so would form a cluster of defectors. In the centre of this cluster, the defectors would not benefit from the cooperation and would thus have to compete with the sensitive competitors. By directing the benefit of cooperation mainly to the cooperators, invasion of defectors should be slowed down, if not prevented.

During the time course of the experiment, colicin production decreased in both the viscous and nonviscous environments. In agreement with the predictions, average colicin production decreased faster and to a lower level in the nonviscous than in the viscous environment. Defectors not producing but still resistant

to the colicin evolved in all the populations from the nonviscous environment, and in two of the five populations from the viscous environment, and are more frequent in the former environment. Three mutations responsible for the absence of colicin production were identified. Bacteria isogenic except for the identified mutations were constructed, and the relative fitness of the ancestral cooperator and mutant genotypes when grown in isolation in the evolutionary environment containing sensitive competitors or when directly interacting with each other in this same environment were calculated. As theoretically expected, these measures suggested that the maintenance of cooperation should be easier in the viscous compared to the nonviscous environment. Surprisingly, further competition experiments revealed that the relative fitness between cooperators and defectors is not affected by the environmental viscosity as imposed in our system. This may be because, in the present system, the viscous environment strongly restricts bacterial movement, but not necessarily the diffusion of the common good. These results are discussed.

Material and Methods

THE BACTERIAL STRAINS

The colicogenic strain used in the present study is *E. coli* W3110 carrying the natural nonconjugative plasmid pcolE8 (strain hereafter named ColE8). This strain produces colicin E8, a toxin cleaving the DNA of target bacteria. ColE8 is *ara*⁺. Two other strains of *E. coli* B are used. REL 606 (hereafter named Comp, Lenski et al. 1991) was used as competitor sensitive to the colicins. The Comp cells are *ara*⁻ and do not carry any plasmid. REL 607 (*ara*⁺, Lenski et al. 1991) was used as a homogeneous genomic background for the different pcolE8 genotypes.

EXPERIMENTAL EVOLUTION

The experimental design of the evolution experiments are illustrated in Figure 1A.

Experimental evolution in viscous environment

ColE8 and Comp were grown overnight on 10 mL DMga (Spencer et al. 2007). To initiate the evolution experiment, five ColE8 (ColE8_1S to ColE8_5S) populations were initiated by spreading 200 μ L of Comp and 50 μ L of ColE8 on tetrazolium plates (TT plates, 1 g.L⁻¹ yeast extract, 5 g.L⁻¹ NaCl, 10 g.L⁻¹ Tryptone, 16 g.L⁻¹ agar, Levin et al. 1977). After 24 h \pm 2 h of growth at 37°C, grown plates were printed on a platform covered with sterile velvet, 200 μ L of an overnight Comp culture were spread on a fresh TT plate (TT_{Comp}) and this plate was pressed on the velvet, taking care to preserve orientation. To prevent the evolution of resistant Comp, after three 24 h \pm 2 h growth periods on TT_{Comp}, each of the five populations was transferred to two minimum arabinose plates (MA plates, Lenski 1988). Because of their

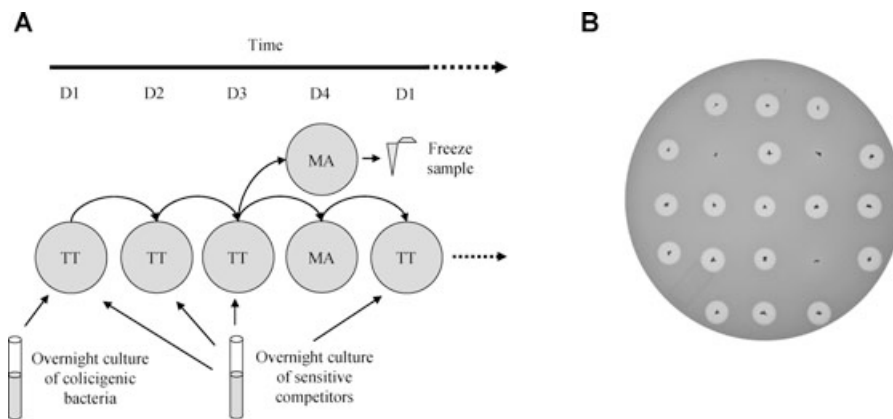


Figure 1. Experimental design. (A) The experimental evolution follows a four-day unit, three days (D1, D2, D3) of growth on TT medium inoculated with 200 μ L of overnight culture of competitors and one day on MA medium (see Materials and Methods). (B) Example of a TT_{assay} plate after incubation.

ara⁻ genotype, Comp are unable to grow on MA plates. After 24 h \pm 2 h of growth at 37°C, the first MA plate was used to transfer the population to a fresh TT_{Comp} and the second plate to freeze a sample of the population coming from a given square of 4 \times 4 mm.² The evolution experiment was continued following this four-day unit (three days on TT_{Comp} and one day on MA). At the beginning of the evolution experiment, we estimated the number of generations of ColE8 to be about 25 per four-day unit (data not shown).

Experimental evolution in nonviscous environment

Experimental evolution was similar to the one on solid medium except for: (1) The experiments were carried out on plates containing 15 mL of medium; (2) the media did not contain agar and TTC; (3) each day 50 μ L of culture was transferred to fresh medium and incubated with an agitation of 30 rpm; and (4) each population was transferred to only one MA plate on the fourth day of the four-day unit. Five ColE8 (ColE8_1U to 5U) populations were initiated. At the beginning of the evolution experiment, we estimated the number of generation to be about 35 per four-day unit (data not shown).

ESTIMATING COLICIN PRODUCTION

Assay plates

To assess colicin production, sterile toothpicks were used to transfer 21 colonies to 21 equally spaced spots on TT_{assay} plates (20 mL per plate of TT medium, containing 10 g.L⁻¹ of agar, and inoculated, before pouring the plates, with 10 mL.L⁻¹ of overnight Comp, Fig. 1B). After incubation overnight at 37°C these plates display a homogeneous dark red background due to the growth of Comp bacteria. Where colicigenic bacteria were transferred, a red spot indicating the growth of the bacteria is surrounded by a clear circular area (Fig. 1B). This area corresponds to the zone into which colicin diffused and killed all the Comp. The bigger the

lysis area, the more colicin is produced. To quantify this production, we took pictures of each assay plate, and used the software ImageJ version 1.36 b (<http://rsb.info.nih.gov/ij/>) to measure the number of pixels corresponding to the lysis area.

Population level production of colicin

To estimate the evolution of the overall level of colicin production in each of the populations, seven samples frozen at different time points during the evolution experiment were grown on 3 mL Lb, for 5 h. Sterile toothpicks were then used to inoculate 21 equally spaced colonies on a TT plate. For each population, seven TT plates were each inoculated with three times the seven time points (21 replicates per time point). After overnight growth at 37°C, each colony was transferred to TT_{assay} plates with a one to one correspondence. Plates were then incubated at 37°C overnight. For each population the mean colicin production at the time point t (C_t) was calculated as: $C_t = (\sum(N_{t,p}/(\text{mean } N_{0,p}))) / 21$ where $N_{t,p}$ is the number of pixels corresponding to the colicin diffusion area at the time point t and for the assay plate p and mean $N_{0,p}$ being the mean number of pixels corresponding to the ancestral colicin diffusion area for the assay plate p . This transformation allows to set the ancestral colicin production to 1.00 and to correct for the error in colicin production measure due to intrinsic differences between plates (some plates tend to allow a better colicin diffusion than others; data not shown). For each population, the correlation between colicin production and time points was tested using Spearman's Rho. Using t statistics, we tested if at the end point of the evolution experiments change in colicin production was different between pcolE8_S and pcolE8_L populations. The results of this experiment are reported in Figure 2.

Colicin production at the clonal level

To estimate the distribution of colicin production in the populations, frozen samples were grown on 3 mL Lb for 5 h, diluted,

plated, and incubated (37°C, overnight) on TT plates to obtain isolated colonies. These colonies were then transferred to TT_{assay} plates. Distribution of colicin production was estimated for all the populations at the endpoint of the evolution experiments and for the ancestral populations by transferring, for each population, 147 colonies to seven TT_{assay} plates and incubating the plates (37°C, overnight). For each colony, the colicin production (C) was calculated as $C = (N_{c,p}/\text{mean}N_{c,p}) \times C_t$ where $N_{c,p}$ is the number of pixels corresponding to the colicin diffusion area for the colony c on plate p . The distribution of colicin production was established for each population. Difference in the variance of the distribution of colicin production between the populations at the end points of the evolution experiments and the ancestral populations were tested using Brown–Forsythe tests. The results of this experiment are reported in Figure 3.

IDENTIFICATION OF MUTATIONS

Starting from the partial sequence of the ancestral *pcolE8* plasmid available (Uchimura and Lau 1987; Toba et al. 1988), we sequenced the whole plasmid. At the end of the evolution experiments, from each population, the colicin E8 operon of several clones reflecting the distribution of colicin production was amplified using the primers col8F4 (CCGGATTATCTCTCCGTC) and R4 (TGCGTCAGAATCGTTTTTCAG) and sequenced using the primers col8F4, R2 (GCACTTAATGCAGCATCAGC), and F1 (TAAGCAGGCTGCATTTGATG). For each mutation identified, the whole plasmid of one clone was sequenced. The sequence of the ancestral *pcolE8* has been submitted to genebank (accession number FJ985252). The mutations are identified on Figure 4.

ASSESSING COOPERATION AND DEFECTION IN PCOLE8 POPULATIONS

To obtain the various *pcolE8* genotypes in the same genomic background, ancestral and mutant *pcolE8* plasmids were isolated and used to transform REL607. The phenotypic effects of the mutations in this common genomic background were assessed on TT_{assay} plates. To assess for cooperation and defection in the *pcolE8* system, transformed REL607 were grown overnight in 15 mL TT. Final optical density at 600 nm did not vary from one genotype to another (data not shown). Final cell concentration was thus considered to be the same for the different genotypes. Pairwise competitions were performed between each of the *pcolE8* genotypes in the REL607 genomic background and the Comp by mixing and incubating 25 μ L of the *pcolE8* genotypes overnight culture with 225 μ L of Comp overnight culture in 15 mL TT (37°C/30 rpm). Three-way competitions were also performed in 15 mL TT (37°C/30 rpm) between each of the mutant *pcolE8* genotypes (25 μ L), the ancestral *pcolE8* genotype (25 μ L), and Comp (200 μ L). After 24 h of incubation, cultures were diluted and plated on tetrazolium-arabinose (TA) agar plates (Levin et al.

1977) and incubated at 37°C. As REL607, but not Comp, is able to metabolize arabinose, strains carrying *pcolE8* plasmids and Comp were distinguished by the color of colonies on TA plates, red for Comp and white for REL607. To distinguish between ancestral and mutant *pcolE8* genotypes in the three-way competitions, for each competition white colonies were transferred to seven TT_{assay} plates (147 colonies) and the genotype mutant or ancestral was assigned according to the presence or absence of a lysis area. Each competition experiment was performed four times. The frequencies of the *pcolE8* genotypes and sensitive competitors after the pairwise and three-way competitions were used as a proxy for the relative fitness. These results are presented in Figure 5.

COMPETITION BETWEEN ANCESTRAL AND MUTANT GENOTYPES IN THE TWO ENVIRONMENTS

Invasion experiments between each of the identified mutants and the ancestral *pcolE8* genotype in the REL607 genomic background were performed. REL607 bacteria carrying the ancestral and mutant *pcolE8* were grown overnight. Initial populations were obtained by mixing mutant and ancestral genotypes in proportions 0.9/0.1 and 0.1/0.9 by volume. In three replicates, 50 μ L of these initial populations were spread and propagated on the viscous and nonviscous environments in the presence of 200 μ L of Comp, as during the evolution experiments, during 12 days (three four day units). For each population, the proportion of mutant and ancestral genotypes at the beginning and the end of the invasion experiment was estimated using 105 colonies as in the procedure described above to estimate the clonal colicin production. The results of this experiment are reported in Figure 6.

Another kind of pairwise competition experiments between the ancestral and each of the *pcolE8* mutants were performed on agar plates. Fresh TT plates were inoculated with 66 equally spaced 5 μ L drops of overnight culture and incubated overnight at 37°C. For each pairwise competition four replicates were performed, two initiated with 17 drops of ancestral genotypes and 59 drops of mutant and two with 59 drops of mutant and 17 drops of ancestor. The initial position of the drops was generated randomly using Microsoft Excel (Microsoft, Redmond, WA) random number generator. A single initial position pattern was used, the replicates initiated with a majority of ancestor being the opposite of the ones initiated with a majority of mutants (preliminary experiments did not indicate any influence of the initial position of the drops). Every 24 h, keeping the spatial structure on each plate by using sterile velvets, these plates were transferred to a fresh TT plate until the clusters of bacteria initiated with the 5 μ L drops touched each other. Transfers were then continued as during the evolution experiment (three days on TT plates, one day on MA plates) during six days. Each day, plates were first transferred to a fresh plate to continue the competition experiment and then to a TT_{assay} plate to visualize the movement of producers and

nonproducers clusters during the competition experiment. After 24 h at 37°C, the TT_{assay} plates were photographed. On the TT_{assay} plates, the presence of colicin released by the producers is identified by clear areas (the Comp embedded in the plate are killed by the colicin), whereas the presence of nonproducers is identified by dark red areas (the Comp embedded in the plate grow). This competition experiment was performed in parallel with and without Comp added to the TT plates prior to velvet transfer. The results of this experiment are reported in Figure 7.

Results

EVOLUTION OF COLICIN PRODUCTION

Ten populations of colicigenic *E. coli* bacteria initiated by a single clone evolved in two different environments containing competitors susceptible to the toxin (Fig. 1A). Five of these populations evolved during about 1200 generations in a viscous environment (a solid culture medium containing agar; populations ColE8_1S to ColE8_5S). The other five evolved during about 420 generations in a nonviscous environment (the same culture medium without agar; populations ColE8_1U to ColE8_5U). Figure 2 shows, for each population, the evolution of colicin production during the evolution experiment as estimated by measuring colicin production at seven equally spaced time points during the experiment (every 70 generations for the populations evolving in the liquid environment, and every 200 generations in the solid environment). In all the populations, average colicin production decreased during the time course of the evolution experiment (Table 1). After 1200 generations in the viscous environment, the mean colicin production of the five populations was $76 \pm 3\%$ of the ancestral colicin production ($t = 7.71$, $df = 8$, $P < 0.0001$). After 420 generations in the nonviscous environment the mean colicin production was $39 \pm 6\%$ of the ancestral production ($t = 9.88$, $df = 8$, $P < 0.0001$). The evolution of colicin production in the two environments seems very different, with a slow regular decrease in the viscous environment compared to a fast decrease after 200 generations in the nonviscous environment (Fig. 2). The mean colicin production after 420 generations in the nonviscous environment is lower than after 1200 generations in the viscous environment ($t = 5.34$, $df = 8$, $P = 0.0009$).

DISTRIBUTION OF COLICIN PRODUCTION

Figure 3 shows the distribution of colicin production for the ancestral clone and at the end of the evolution experiment for the 10 evolved populations. For almost all of the evolved populations, the variance of the distribution is larger than in the ancestral population (Table 2), indicating the probable coexistence of several genotypes differing in their colicin production. Moreover, in two of the five populations that evolved in the viscous environment (colE8_1S and colE8_4S) and in all the populations

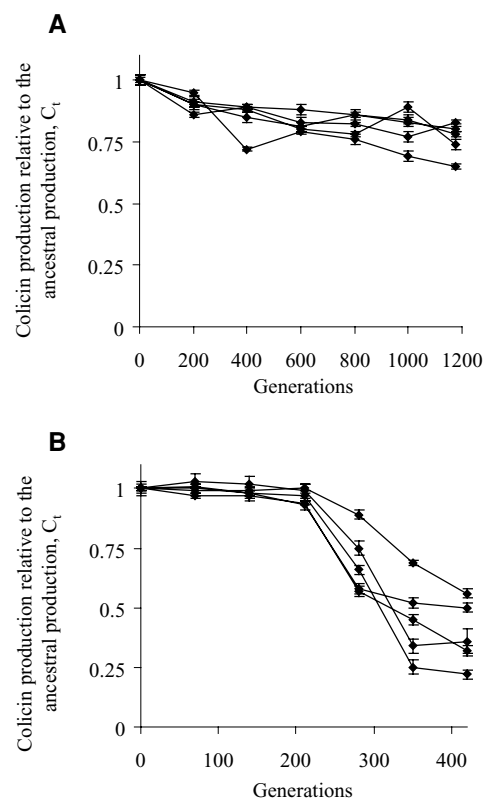


Figure 2. Evolution of total colicin production (C_t) during the evolution experiments in (A) the five populations that evolved in the viscous environment (populations ColE8_1S to 5S) and (B) the five populations that evolved in the nonviscous environment (populations ColE8_1U to 5U). SEM are indicated.

that evolved in the liquid environment, some bacteria seem to produce virtually no colicin. These very low producers (defined by a colicin production relative to the ancestor $< 30\%$) are less frequent in the viscous environment (mean population

Table 1. Correlation between colicin production and number of generations since the beginning of the evolution experiment. The Colicin production at the end of the evolution experiment, relative to the ancestral production is indicated, as well as the results of the spearman correlation test.

Population	End production \pm SEM	Spearman Rho	<i>P</i> -value
pcolE8_1S	0.74 \pm 0.01	-0.79	0.036
pcolE8_2S	0.78 \pm 0.01	-0.82	0.023
pcolE8_3S	0.83 \pm 0.01	-0.81	0.027
pcolE8_4S	0.80 \pm 0.01	-1.00	0.00
pcolE8_5S	0.65 \pm 0.02	-0.89	0.007
pcolE8_1U	0.56 \pm 0.02	-0.84	0.02
pcolE8_2U	0.32 \pm 0.02	-0.99	<0.0001
pcolE8_3U	0.50 \pm 0.02	-0.99	<0.0001
pcolE8_4U	0.22 \pm 0.02	-0.96	0.0005
pcolE8_5U	0.36 \pm 0.05	-0.86	0.01

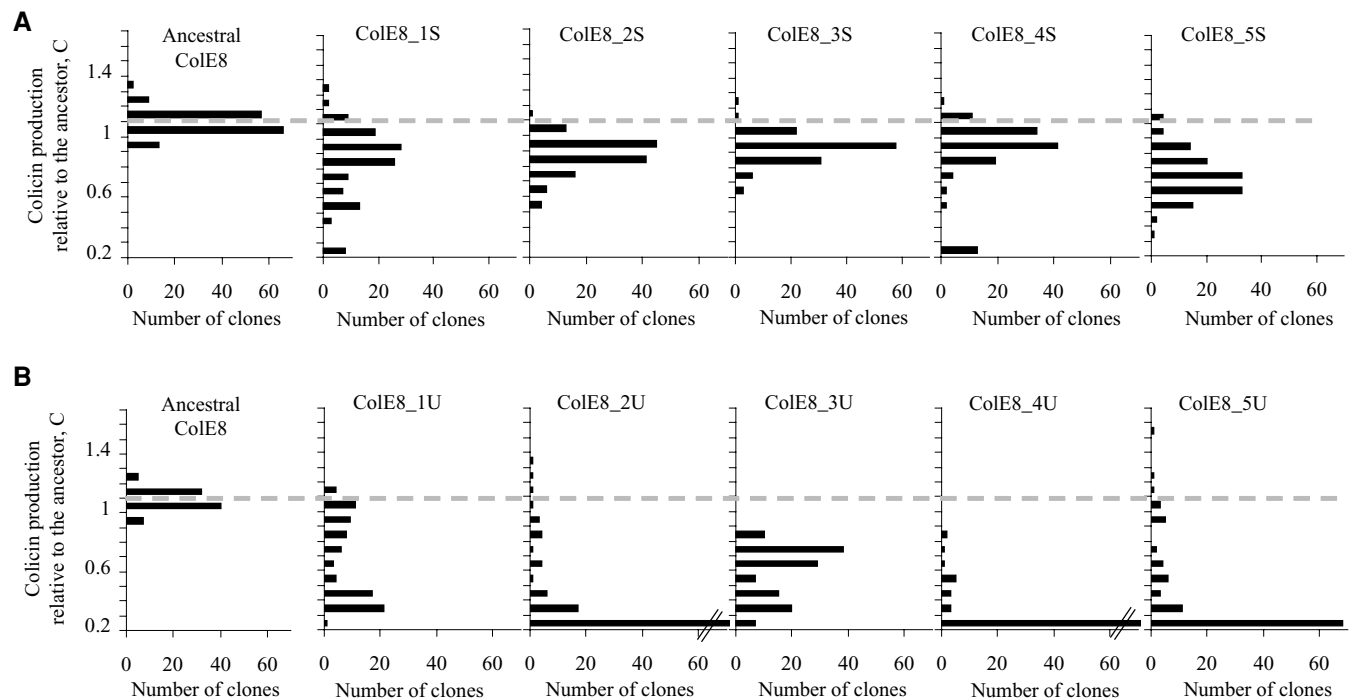


Figure 3. Distribution of colicin production at the beginning (Ancestral ColE8) and the end of the evolution experiment in (A) the viscous environment (populations ColE8_1S to 5S), and (B) the nonviscous environment (populations ColE8_1U to 5U). The dotted lines indicate the mean ancestral colicin production.

frequency = 0.03 ± 0.02) than in the nonviscous environment (mean population frequency = 0.59 ± 0.20 ; $t = 3.76$, $df = 8$, $P = 0.006$).

MUTATIONS CAUSING A LACK OF COLICIN PRODUCTION

To investigate the genetic modifications associated with the decrease in colicin production, several clones (between two and

Table 2. Brown–Forsythe test for unequal variances between the distribution of colicin production at the end and the beginning of the evolution experiment.

Population	Standard deviation	F-Ratio	df	P-Value
pcolE8_Anc	0.080			
pcolE8_1S	0.246	64.5	271	<0.0001
pcolE8_2S	0.118	7.7	271	0.006
pcolE8_3S	0.096	0.5	267	0.45
pcolE8_4S	0.247	22.9	272	<0.0001
pcolE8_5S	0.156	26.2	271	<0.0001
pcolE8_Anc	0.073			
pcolE8_1U	0.284	82.9	166	<0.0001
pcolE8_2U	0.228	10.4	208	0.0014
pcolE8_3U	0.179	35.7	208	<0.0001
pcolE8_4U	0.112	3.5	208	0.063
pcolE8_5U	0.27	15.9	187	<0.0001

eight per population) displaying different levels of colicin production were isolated from each evolved population. The colicin operon of these clones was sequenced and mutations were identified in the clones displaying a very low level of colicin production in the populations ColE8_1S, ColE8_4S (solid environment), and in the five populations that evolved in the liquid environment (ColE8_1U to 5U). In the population ColE8_1S, the five very low producers sequenced display an IS5 insertion in the C-terminal region of the colicin gene (mutation M1, Fig. 4). Three of the six very low producers sequenced in the population ColE8_4S as well as all the very low producers from the populations ColE8_1U to 5U (i.e., from the populations that have evolved in the nonviscous environment, from which three clones were sequenced per population) have another IS5 insertion located 9 bp downstream of the mutation M1 (mutation M2, Fig. 4). The three other very low producers sequenced from the ColE8_4S population have a substitution in the SOS boxes (mutation M3, Fig. 4). The mutation M3 is a substitution of a T by a G at a site that is conserved in the SOS boxes of all the genes regulated by LexA (Lewis et al. 1994). This mutation may prevent the dissociation of LexA from the SOS boxes, allowing the constitutive repression of colicin production. In the mutations M1 and M2, the insertion of the IS5 causes the addition of a codon stop right at the beginning of the insertion, which prevents the production of a functional colicin protein. No other mutations were identified. To demonstrate that these three mutations are responsible for the lack of colicin production,

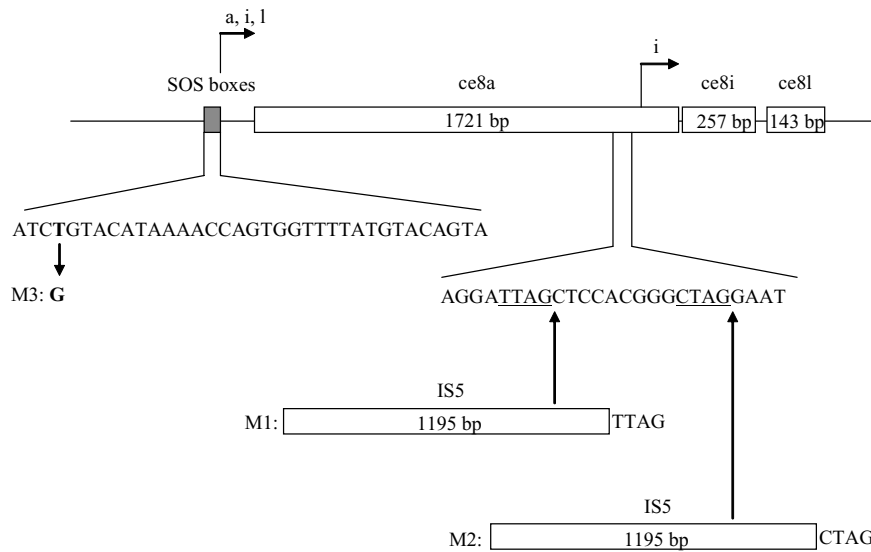


Figure 4. Organization of the colicin E8 operon. Positions of the SOS boxes, colicin (*ce8a*), immunity (*ce8i*), and lysis (*ce8l*) genes are indicated, as well as the promoter controlling the expression of *ce8a*, *ce8i*, and *ce8l* (a, i, l), and the constitutively active *ce8i* promoter (i). The position of the IS5 insertion corresponding to the mutations M1 and M2 (the target sequence duplicated during the insertion is underlined), as well as the position of the mutation M3 (a substitution of a T by a G) in the SOS boxes are indicated.

the ancestral plasmid, and three derived plasmids containing the mutations—M1, M2, and M3—were entirely sequenced. This sequencing did not detect any other mutation. These plasmids were transformed in the bacteria REL607. When assessed on TT_{assay} plates, the bacteria carrying the mutations M1, M2, and M3 produce a very small lysis area or no lysis area at all, whereas the bacteria carrying the ancestral plasmid display a large lysis area (data not shown).

RELATIVE FITNESS

The mutants M1, M2, and M3 seem to produce very little colicin, if at all, compared to the ancestral genotype. The conceptual starting point for our evolution experiments was that colicin is a common good, killing the sensitive competitors and thus increasing the availability of spatial and nutritional resources for bacteria that are immune to colicin. However, the production of this common good comes at a cost to the colicigenic bacteria, because the fraction of the bacteria population that actually produces colicin essentially commits suicide to release the toxin into the environment. The mutants M1, M2, and M3 thus seem to take advantage of the destruction of the sensitive competitors without paying the cost associated with the toxin production. To assess the cooperator-defector relationship between ancestor and mutant genotypes, competition experiments were performed between: (1) REL607 bacteria carrying the ancestor *pcolE8* and sensitive competitors (pairwise competition); (2) REL607 bacteria carrying either *pcolE8M1*, *pcolE8M2*, or *pcolE8M3*, and sensitive competitors (pairwise competition); and (3) REL607 bacteria carrying the ancestor *pcolE8*, REL607 bacteria carrying *pcolE8M1*, *pcolE8M2*, or *pcolE8M3*, and sensitive competitors (three-way competition). Relative fitness was estimated from these competition experi-

ments, as presented in Figure 5. When competing with Comp only, the relative fitness of the ancestral plasmid (1.00 ± 0.00 , as indicated by the height of the corresponding bar in Fig. 5) is higher than that of *pcolE8M1* (0.13 ± 0.02 , $t = 36.38$, $df = 6$, $P < 0.0001$) or *pcolE8M2* (0.12 ± 0.02 , $t = 55.76$, $df = 6$, $P < 0.0001$), but not than the relative fitness of *pcolE8M3* (1.00 ± 0.00 , $t = 0.19$, $df = 6$, $P = 0.85$). The relative fitness of *pcolE8M1* and M2 bacteria is enhanced by the presence of the ancestral *pcolE8* bacteria (0.67 ± 0.04 , $t = 10.47$, $df = 6$, $P < 0.0001$ for *pcolE8M1*; 0.48 ± 0.01 , $t = 16.09$, $df = 6$, $P < 0.0001$ for *pcolE8M2*). On the contrary, the relative fitness of *pcolE8M3* bacteria is smaller when competing with both Comp and ancestral *pcolE8* (0.74 ± 0.02 ,

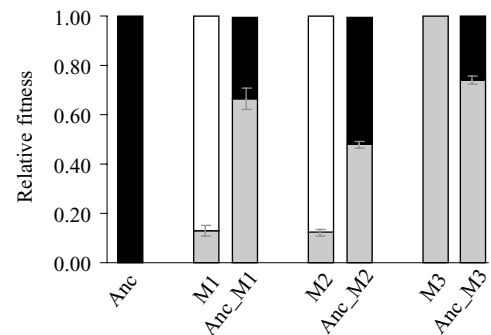


Figure 5. Assessing cooperation and defection. Relative fitness of ancestral genotype (*pcolE8*, in black), sensitive competitors (Comp in white), and mutants (*pcolE8M1*, M2, or M3, in grey) during pairwise competitions between: *pcolE8* and Comp (Anc); M1 and Comp (M1); M2 and Comp (M2); M3 and Comp (M3); and during three-way competitions *pcolE8*, M1, and Comp (Anc_M1); *pcolE8*, M2, and Comp (Anc_M2); *pcolE8*, M3, and Comp (Anc_M3). SEM are indicated.

$t = -14.73$, $df = 6$, $P < 0.0001$). Because the growth of the mutants M1 and M2 is enhanced by the presence of the ancestral genotype, and the growth of the ancestor is reduced by the presence of these mutants, the mutants M1 and M2 can be considered to be defectors exploiting the cooperative investment made by the bacteria carrying the ancestral plasmid. The bacteria carrying the mutation M3 cannot be considered defectors because their performance when competing against the sensitive competitors is not enhanced by the presence of the ancestral genotype. On the contrary, despite producing very little colicin on the phenotypic assays, no sensitive competitor was detected after 24 h of competition with *pcolE8M3*, suggesting that this mutant still produces enough colicin to kill all the sensitive competitors.

In a nonviscous environment, interaction between the bacteria is global, and as a result, the relative fitness of the ancestral plasmid, mutants, and Comp, should be directly given by the result of the three-way competition experiments. In a viscous setting, interactions should be more local. In the same population some bacteria should interact as in the three-way competitions, and others as in the pairwise competitions, depending on the composition of their spatial neighborhood. For example, if we consider the interaction between the ancestral genotype and either *pcolE8M1* or *pcolE8M2*, we expected the ancestral genotype to do better in the viscous environment compared to the nonviscous environment, because the relative fitness of the ancestral genotype is higher than that of the mutants in pairwise competitions with the sensitive competitors. Thus, the ancestor should do better than either mutant in spatial regions in which only one strain is present in addition to the sensitive competitors. On the other hand, in spatial regions in which all three strains (i.e., the ancestor, one of the mutants, and the sensitive competitor) are interacting, the ancestor should do worse than the mutant. Because the whole spatially structured population should have both regions with only two strains (either the ancestor or the mutant plus the sensitive competitor) and regions with three interacting strains, the ancestor is therefore predicted to do better overall in viscous populations than in well-mixed populations (in which ancestor, mutant, and sensitive competitors are always involved in three-way interactions). For analogous reasons, if we consider the interaction between the ancestral genotype and *pcolE8M3*, we expected *pcolE8M3* to invade the ancestral genotype in both viscous and nonviscous environments, because the relative fitness of the mutant is the same as the ancestral genotype in the pairwise competitions, but higher in the three-way competitions (Fig. 5).

COMPETITIONS BETWEEN ANCESTRAL AND MUTANTS GENOTYPES IN THE TWO ENVIRONMENTS

To test these predictions, competition experiments were performed between the ancestral and mutant genotypes (in the

REL607 genomic background) in the viscous and nonviscous evolutionary environment in the presence of sensitive competitors. Competitions were initiated with 0.9 and 0.1 of ancestral genotype. Results are presented in Figure 6, and indicate that: (1) contrary to what was expected, environmental viscosity does not seem to influence the outcome of competition, although there seems to be a small difference between viscous and nonviscous environment when the ancestor is competing with the M2 mutant; (2) the initial frequency of ancestor does not seem to affect the outcome of competition; (3) the dynamics of competition strongly depends on the mutant involved, with M1 probably being able to slowly invade the ancestor but displaying a large variability among replicates, M2 seemingly coexisting with the ancestral genotype, and M3 quickly invading and almost driving the ancestor to extinction (see Table S1 for statistical analyses).

The competition experiments as performed above were initiated with a mixture of ancestor and mutant. At any point in space in the viscous environment the competition was initiated by a mixture of 0.9 of one type and 0.1 of the other type. Thus,

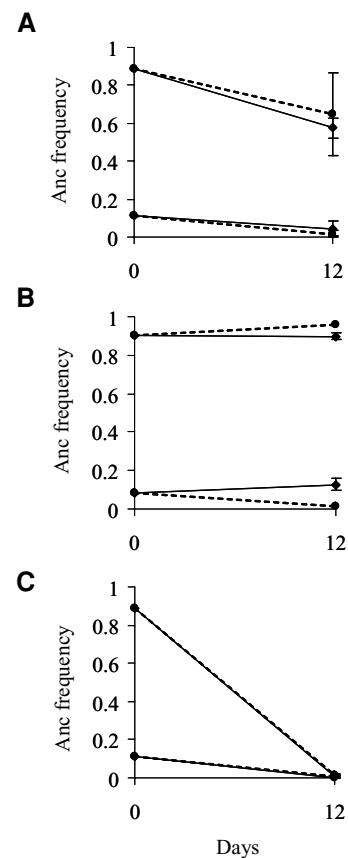


Figure 6. Invasion experiments between (A) *pcolE8* and *pcolE8M1*, (B) *pcolE8* and *pcolE8M2*, (C) *pcolE8* and *pcolE8M3*, in the viscous (full lines) and nonviscous (dotted lines) environments. Mean *pcolE8* frequencies (Anc frequency) are indicated at the beginning and the end of the competitions. SEM are indicated.

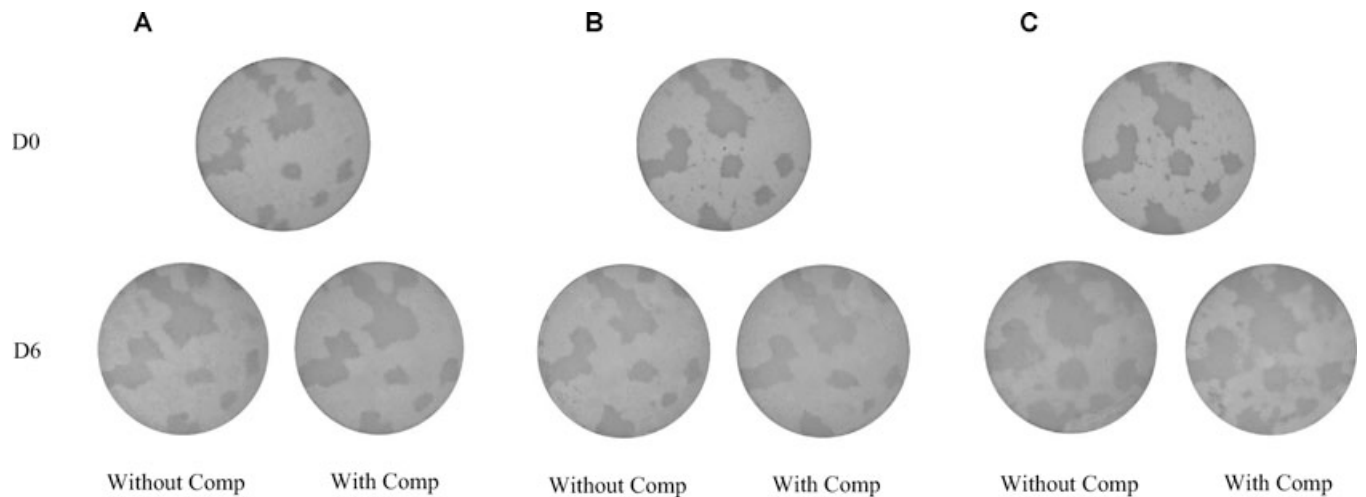


Figure 7. Competition experiment initiated with clusters of *pcolE8* and (A) *pcolE8M1*, (B) *pcolE8M2*, and (C) *pcolE8M3* and performed in the viscous environment with (with Comp) and without (without Comp) sensitive competitors. Results are presented at the beginning (D0) and after six days of competition (D6) for only one replica but are similar for the others. The dark areas indicate the presence of the mutants and the clear areas the presence of the ancestral genotype *pcolE8*.

the starting populations were locally well-mixed. The advantage of cooperation in a viscous environment should be linked to the presence of clusters of cooperators and defectors. Moreover, the benefit of the cooperation itself should help prevent the invasion of the cooperator clusters by defectors. To investigate how the existence of clusters of cooperators and defectors affect competition between the two, experiments were initiated in the viscous environment using clusters of ancestors and mutants in the REL 607 genome. To estimate how the benefit of cooperation influences the fate of the competition, experiments were performed in parallel with and without the sensitive competitors. Mutual invasion of ancestor and mutant clusters was visually followed during six days. The results are illustrated in Figure 7. Once again, the results of the experiment strongly depend on the mutant genotype. M1 mutants slowly invade the ancestor clusters, M2 clusters stay relatively stable, and M3 clusters invade faster during the time course of the competition. Surprisingly, the result of the competition experiment is very similar when performed with or without the sensitive competitors, indicating that the benefit of cooperation, which is to kill off competitors, has no detectable influence on the fate of competition between ancestor and mutant genotypes. It should also be noted that, even though their insertion site is just 9 bp apart, the two IS5 mutants do not have the same fitness when competing with the ancestral genotype.

Discussion

Colicigenic bacteria cooperating to kill competitors sensitive to their toxin were propagated in a viscous and nonviscous environ-

ment for several hundred generations. During the time course of the experiment, the average degree of cooperation, measured as the amount of colicin produced, decreased in both environments. At the end of the evolution experiment the level of cooperation was higher in populations that evolved in viscous environments, even though they evolved for more than twice the number of generations. Bacteria producing very little colicin, but still resistant to the toxin, were identified at relatively low frequency in two of the populations that evolved in the viscous environment and at a higher frequency in all five populations that evolved in the viscous environment. By sequencing the plasmid encoding the colicin production, three mutations responsible for the absence of colicin production were identified. One of these mutations is a substitution at the binding site of the repressor of colicin production (M3). The two other mutations are due to an insertion of the insertion sequence IS5 in the coding region of the colicin gene (M1 and M2). These IS5 mutations were identified in seven different populations. The repetitive occurrence of these mutations may seem surprising, but the mutation rate due to insertion sequence is supposed to be high (Schneider and Lenski 2004), and with 23 IS5 copies mapped on the *E. coli* W3110 chromosome, this IS element is one of the most common in this bacteria (Umeda and Ohtsubo 1990). The mutation M2 occurred in six different populations. The target motif for IS5 insertion is YTAR, with CTAG being the preferred site (Mahillon and Chandler 1998), and the site where the IS5 insertion occurred repeatedly is the only CTAG motif present in the colicin gene-coding region, probably making this mutation the most likely to occur. In natural populations, numerous bacteria are resistant to colicins without carrying the plasmid, but to our knowledge, the mutants described above are

the first ones resistant to, but not producing the toxin because of plasmidic mutations.

The effect of the mutations on the colicin production was assessed by placing the three mutants as well as the ancestral plasmids in a common genomic background. Competition experiments demonstrated the cooperator–defector relationship between the ancestral genotype and the two IS5 mutants (M1 and M2) by showing that the ancestral genotype performs better than the mutants when competing with sensitive competitors only, and that IS5 mutants tend to exploit the ancestral genotype when mutant and ancestors are growing together in the presence of sensitive competitors. However, the third mutant (M3) should not be considered a defector as it performs better against the competitors when the ancestral genotype is absent, indicating that this mutant probably still produces some toxin. By producing very little colicin, this mutant seems to maximize the benefit of cooperation (the allocation of the resource to the colicigenic bacteria) while minimizing its cost (the probability of suicide). It seems to give an optimal cooperating strategy and should invade and replace the ancestral cooperator genotype. As expected, the competition experiments indicate that the M3 mutant is always able to invade the ancestral genotype. Despite the fact that this mutant probably still produces some colicin, and thus pays the cost of its production, this mutant always invades the ancestor faster than the IS5 mutants that do not produce any active colicin. This can only be explained by the type of mutation involved. Although the IS5 mutations may prevent the lysis of the cells, and the production of an active form colicin, some inactive colicin may be produced. Moreover, the IS5 mutations involve the insertion of 1.2 kb in all the pcolE8 plasmids (about 20) of a given bacteria, representing an increase of about 20% in plasmid size. Such a mutation might decrease the fitness of bacteria by increasing the intrinsic cost of carrying the plasmid, and by producing an inactive product. Despite its strong advantage when competing against the ancestral genotype, the M3 mutation occurred only in one population during the evolution experiment, suggesting that such a mutation is rare.

According to pairwise and three-way competition experiments, cooperation should be favored in a viscous compared to nonviscous environment, because cooperators should have preferential access to the public good produced (the toxin). But surprisingly, the relative fitness of ancestral and mutant genotypes was not affected by environmental viscosity. Also surprisingly, when the environment was manipulated to remove potential benefits of cooperation (by removing the Comp), the outcome of competition was not affected either. On the one hand, these results are easily interpreted for the competition experiments involving the M3 mutant, as this mutant cannot be considered as a defector and probably outcompetes the ancestral genotype in any situation. On the other hand, the two other mutants (the IS5 mutants M1 and M2)

seem to exploit the altruism of the ancestral genotype. Invasion of the ancestral genotype by these two mutants was thus expected to be favored in the nonviscous environment, and when the benefit of cooperation is removed. Theoretical studies have pointed out that cooperation is not always favored by environmental viscosity, as under some conditions the increase in local competition between cooperators should exactly cancel the benefit of cooperation (reviewed in Queller 1992). However, this result only applies if the population regulation is locally inelastic (West et al. 2002), i.e., if cooperation does not increase the number of individuals, a condition clearly not met in the present environment, because in the present system cooperation increases the amount of resources available to the colicigenic bacteria and hence the number of colicigenic bacteria. Another particularity of the present system may explain why competition experiments do not seem directly affected by the cooperator–defector relationships. When colicin production is assessed on agar plates, the lysis area surrounding the colicigenic bacteria clearly shows that colicin diffuses much faster than the bacteria themselves. This indicates that the environment is viscous in terms of bacterial dispersal, but not in terms of colicin diffusion. The benefit of cooperation is not restricted to the cooperators only but is available to spatially distant cells. In this situation, a selfish mutant invading a population of cooperators, and forming clusters of defectors, would benefit from a “buffer zone” in which neither the cooperators nor the sensitive competitors would be present. Such a zone would separate the areas in which defectors are competing on the one hand with sensitive competitors and on the other hand with cooperators. This would buffer the fitness difference between cells in clusters of cooperators and clusters of defectors, potentially removing any effect of environmental viscosity on the maintenance of cooperation. In other words, cooperation is not favored in the viscous environment, essentially because the viscous environment is not really viscous with respect to the distribution of the public good. Thus, the relative fitness of the SOS box mutant, IS5 mutants, and ancestor genotype is not affected by environmental viscosity as imposed in our system, and may instead simply reflect the relative costs associated with each genotype: the cost of carrying the insertion and producing inactive colicin for the IS5 mutants, the cost of abundant production of colicin for the ancestor, and the cost of producing very little colicin for the SOS box mutant. We note that in natural populations, bacterial density is typically much lower than in the experimental setting used here. In nature, the combination of small density and spatial viscosity may thus help directing the benefit of cooperation to cooperators only and to maintain cooperation among colicigenic bacteria.

Long-term evolution was very different in the viscous and nonviscous environments. If the cooperator–defector relationship is not affected by the amount of viscosity, what could account

for this difference? During the evolution experiment, competitive interactions between genotypes were probably very different in the two environments, with the nonviscous environment allowing a global competition between all the genotypes present in a given population and the viscous environment promoting very local competition between few genotypes at each point in space. Global competition is known to increase the speed of adaptation in bacteria (see Perfeito et al. 2008 for example). The scale of competition may thus explain the difference in the evolutionary dynamics between the two environments, with the invasion of the defectors being faster in the nonviscous environment because adaptation is faster in the nonviscous environment. Moreover, differences in the scale of competition between the viscous and nonviscous environment may also explain why the same mutation invades all the populations in the nonviscous environment while several mutations appeared in the viscous environment. The global competition, leading to the direct interaction between a large number of individuals, in the nonviscous environment would tend to always favor the mutation having the higher probability to invade (the probability of invasion being linked to the frequency and the fitness of the mutation), whereas in the viscous environment, the local competition, leading to the direct interaction between a small number of individuals, would give more weight to genetic drift and would allow less likely mutations to appear and invade the population locally.

Conclusions

In the present system, the production of colicin is a cooperative act allowing, by interference competition, the allocation of more resource to bacteria resistant to the toxin. Mutants that are resistant against but not producing the toxin exploit the altruism of the colicigenic bacteria. We observed the evolution of cooperation in viscous and nonviscous environments by propagating experimental populations for several hundreds of generations. Because viscous environments tend to generate assortment between cooperators, we expected that cooperation is better maintained in viscous environments. Indeed, cooperation remained at a higher level in the viscous than in the nonviscous environment. However, this difference probably reflects the fact that evolution is faster in nonviscous environments, because competition experiments between cooperators and defectors (which evolved during the evolution experiments) revealed that viscosity as imposed in our system does not influence the relative fitness of cooperators and defectors. Most likely, this is because viscosity, while restricting bacterial movement, does not strongly restrict colicin diffusion. Thus, when it comes to cooperation, environmental viscosity should be considered not only in terms of individual movement, but also in terms of the distribution of the public good. If that distribution is not limited enough, cooperators cannot gain the preferential

access to the public good necessary for their dominance, even if they do form spatial clusters.

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Supporting Information

The following supporting information is available for this article:

Table S1: Competition experiments between ancestral and mutant *pcolE8*.

Supporting Information may be found in the online version of this article.

(This link will take you to the article abstract).

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