Bacteria use collective behavior to generate diverse combat strategies

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SUMMARY

Animals have evolved a wide diversity of aggressive behavior, often based upon the careful monitoring of other individuals. Bacteria are also capable of aggression, with many species using toxins to kill or inhibit their competitors. Like animals, bacteria also have systems to monitor others during antagonistic encounters, but how this translates into behavior remains poorly understood. Here we use colonies of *Escherichia coli* carrying toxin-encoding plasmids as a model for studying antagonistic behavior. We show that, in the absence of threat, dispersed cells with low reproductive value produce toxins spontaneously, generating efficient pre-emptive attacks. Cells can also respond conditionally to toxins released by clonemates via autoinduction, or other genotypes via competition sensing. The strength of both pre-emptive and responsive attacks varies widely between strains. We demonstrate that this variability occurs easily through mutation by rationally engineering strains to recapitulate the diversity in naturally-occurring strategies. Finally, we discover that strains that can detect both competitors and clonemates are capable of massive coordinated attacks on competing colonies. This collective behavior protects established colonies from competitors, mirroring the evolution of alarm calling in the animal world.

KEYWORDS

Toxin, colicin, bacteriocin, aggression, competition, bacteria, collective behaviour

INTRODUCTION

The study of aggression and conflict has a long history in ethology and evolutionary game theory [1-3]. In animals, aggression is often associated with sophisticated strategies, whose evolutionary rationale is to ensure that a fight is initiated only when its benefit outweighs its cost [4]. In many species, the decision to enter conflict is based on the assessment of multiple factors, including the availability of resources and the condition of both friends and foe. Extra layers of behavioral complexity are possible in social animals, where altruistic sacrifice occurs and collective behavior is used to coordinate attacks. Well-known examples come from the social insects, including the evolution of specific castes for colony defence [5] and alarm pheromones that coordinate attacks against predators [6]. A key prediction of the game theory of animal conflicts is that natural selection can favour variability in aggressive

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behaviors both within and between species [7]. This is borne out in animal populations, where there is striking diversity in the strategies determining when aggression is initiated, and the length and violence of fights [1, 8].

Bacteria live in dense communities, where their capacity to monitor and interact appropriately with the cells around them is key to their evolutionary success [9]. Consistent with this, bacteria have evolved systems that allow them to assess the density of cells, the intensity of resource competition, and the presence of competitors, and to change their individual and collective behavior accordingly [10-12]. Most bacterial strains also produce antibacterial compounds, like antibiotics, bacteriocins and other types of toxins [13]. These compounds enable them to inhibit competing strains and to secure space and resources [14-18]. Therefore, such molecules can be considered functionally equivalent to the aggressive behaviors of animals [8]. Molecular microbiology has revealed that toxin production is usually controlled by regulatory systems commonly associated with stress responses and cellular damage [19-27]. This has led to the idea that toxins are often upregulated in response to competition [10]. However, given the relative simplicity of bacterial regulatory systems governing the production of toxins, it remains unclear whether the richness in variability and complexity seen in animal aggression strategies is a realistic prospect.

Here we investigate the diversity and complexity of antagonistic behavior in bacteria. We focus our study on a group of prototypic bacterial toxins, the colicins, whose production is controlled by a regulatory system which detects DNA damage (SOS response) [28]. By monitoring the localization of toxin production within bacterial colonies in the presence or absence of competing strains, we show how colicinogenic bacteria modulate their behavior, individually and collectively, in response to competition and to their clonemates' behavior. Additionally, we show that different genotypes display a wide diversity of antagonistic behaviors, which can be generated through a small number of mutations affecting the toxin-encoding locus. Ultimately, our results reveal that, even when using one of the simplest regulatory systems known - based on a single transcriptional repressor - bacteria can generate a remarkable diversity of toxin production strategies, including collective responses akin to those seen in social animals [29, 30].

RESULTS

Spontaneous toxin production is common across colicinogenic strains

Colicinogenic plasmids (Type I) in Enterobacteria encode three proteins relevant to toxin production: an activity protein (the actual toxin), an immunity protein (that interacts with the toxin to prevent it from harming the producer strain) and a lysis protein (which releases the toxin through the permeabilization of the producer's membrane) [31-33]. At the regulatory level, the decision to produce most characterized colicins is thought to lie primarily with LexA, a transcriptional repressor whose main function is to prevent the expression of genes involved in SOS response during normal growth [22, 34, 35]. In agreement with this, we found that the promoter region of approximately 60% of the 176 colicin genes identified in the NCBI plasmid database (Enterobacteria only) contained at least one, but often two, putative LexA binding site(s), whereas potential binding sites for the stress regulators CRP [36, 37], IscR [38, 39] and FUR [40] were present in only 12, 27 and 40% of the promoters, respectively (Figure S1A, File S1 - Tab 1). LexA is, therefore, the most frequent regulator of colicin-like toxins [21, 41]. Interestingly, most of the individual LexA boxes found in colicin promoters are predicted to bind LexA rather weakly (Heterology Index score (HI) > 6) (File S1 - Tab 1), unlike the LexA boxes found in the promoters of typical chromosomal SOS response genes (HI < 5 for *umuD, lexA, sulA, recA, recN, uvrB*) [22, 34]. This consistently

low predicted affinity of LexA boxes in colicin promoters suggests that transcription of these toxins might not be completely repressed, and that colicins could be commonly produced in the absence of DNA-damaging agents. This is in agreement with previous data from some of the well-studied colicins in liquid culture [28, 34, 42, 43] but it is not clear whether the amounts produced are biologically significant and able to suppress other strains in direct competition.

We, therefore, asked if spontaneous toxin production across several colicinogenic strains is sufficient to inhibit competitors. Specifically, we examined whether thirteen LexA-regulated colicins of different toxic activities were produced spontaneously within agar colonies in amounts that inhibit another strain. When incubated overnight, eight of the thirteen colicin producing strains inhibited the growth of a wild-type non-producer spotted nearby at low densities (Figure S1B). For the other five colicins, colonies had to be grown for 12 hours prior to adding the non-producer for growth inhibition to be observed, also consistent with colicins being made at significant levels, but in a manner where established colonies inhibit later arrivals. This suggests that colonies formed by all examined colicin-encoding strains produce toxins spontaneously, in biologically relevant amounts.

We then focused on a subset of five toxins for more detailed study. We chose colicins E2, E7 and E8, which are DNases, and colicins A and K, which have pore-forming activity (see also Table S1). To assess the baseline activity of colicin expression, we first studied wild-type strains harboring GFP reporter plasmids for each of the five colicin promoters (Table S2). While most cells in colonies grown from a single cell (hereafter termed "single-cell colonies") did not express significant amounts of GFP, a small fraction (from 0.1 to 2% depending on the reporter plasmid), dispersed among inactive cells, was highly fluorescent (Figure 1AB). Colicin E2 and E7 reporters were expressed in a larger fraction of the cells than E8 and A reporters, which were, in turn, more active than colicin K. A reporter for the chromosomal gene *umuD*, activated in cells undergoing sustained SOS response, was expressed at frequencies comparable to colicin K [44]. The result we obtained in structured environments can be also reproduced in liquid cultures [22, 45]. We confirmed that these expression patterns are almost entirely dependent on LexA cleavage, except for colicin K, whose expression is known to be controlled also by stringent response, especially in stationary phase [25] (Figure S2A).

In sum, we find that, in colicinogenic colonies, a small fraction of cells which are well-dispersed in the colony, express colicin spontaneously, and that this level of colicin expression is sufficient to inhibit a sensitive competitor (Figure S1B). We, therefore, conclude that colicins are generally consistently produced in the absence of threat, generating what is, from a behavioral perspective, a pre-emptive attack.

Spontaneous colicin production occurs in cells of low reproductive value

To better characterize the interaction of chromosomal SOS response and plasmid-driven toxin production, we constructed a strain harboring a dual reporter plasmid (Table S2), where the promoter of colicin E2 controls the transcription of a GFP gene, and the promoter of the canonical SOS response gene *umuD* controls the transcription of an RFP gene. This allows us to compare the relative activation of stringent SOS response and colicin production at a single-cell level. We found that in single-cell colonies (Figure S2B, top), all cells that were expressing *umuD* (red) also expressed colicin E2 (green), but most cells producing colicin E2 did not express *umuD*. The same held true for liquid culture of BZB1011 cells harboring the dual *colE2*/*umuD*-reporter plasmid; cells active solely for colicin expression were approximately twice as frequent compared to cells that would make colicin while undergoing stringent SOS response (Figure S2B, bottom and S2C). Therefore, colicin promoters are more reactive to low-level DNA damage than stringent chromosomal SOS genes and this leads to

an increase in the number of cells in a colony committed to toxin production. This extra investment poses the question of the rationale of toxin production in the absence of threat.

It has been previously suggested that producing colicin in response to DNA damage ensures that cells with low fitness (reproductive value) are the ones which will end up lysing to release the toxin [10, 46, 47]. However, to our knowledge, this hypothesis has never been empirically tested. To address this, we transformed BZB1011 cells with either the colicin E2 or the *umuD* reporter plasmids (in the absence of any natural colicin plasmid) and recorded cell divisions (Figure 1C). Cells undergoing sustained SOS response were more than six times less likely to divide in a given timeframe than cells in which the *umuD* promoter was inactive. Cells which were reporting colicin expression were only two-fold more unlikely to divide than non-reporting cells. Nevertheless, the difference in short-term fitness between the cells that reported colicin expression and the ones that did not is significant. Since the strains we used harbored only reporter plasmids, this experiment reflects the actual health of the cells which would produce colicin should they contain a colicin plasmid. Hence, we can conclude that LexA regulation identifies a subset of cells that have relatively low immediate reproductive value but that are likely to be healthy enough (to have sufficient "social value" [47]) to produce large amounts of colicin proteins and generate an efficient pre-emptive attack.

Autoinduction generates variability in toxin production behavior during pre-emptive attack

For a subset of colicins with DNase activity, an increase in toxin production (termed "autoinduction") triggered by the presence of colicin molecules in the medium has been observed in liquid cultures [48, 49]. To investigate the potential contribution of autoinduction to the generation of behavioral diversity in combat, we repeated our reporter experiments in colonies with strains that contained both a reporter and its corresponding colicin plasmid (Figure 2). While expression of colicin A, K and E7 did not change dramatically, colicin E2 and E8 showed a large increase in spontaneous expression frequency in colonies, which we confirmed ourselves also for liquid culture (Figure S3) [48, 49]. The high expression levels of colicin E2 and E8 are also associated with increased killing capacity (Figure S1B). Therefore, even though the different toxins may have different potency, the transcriptional regulation of colicins is a predictor of aggression in *E. coli*.

In colonies of colicin E2 and E8 strains, large intensely-fluorescent patches of 10-100 μm in diameter appeared (Figure 2A). This contrasts with the reporter-only experiments where individually fluorescent cells were distributed across the entire colony in the (Figure 1A). Time-series experiments on single-cell colonies of colicin E2 and E8 showed that the colicin-expressing patches dramatically increased in size over time until the center of the colony is almost entirely occupied by colicin-producing cells, suggesting that the spontaneous release of colicins by individual cells induced colicin production in their immediate neighbors, which, in turn, propagated the response in space (Figure S4A). We also found evidence for autoinduction between neighboring cells at the single-cell level (Figure S4B, Video S1), which demonstrates the importance of spatial structure for this amplification event. Finally, we confirmed that autoinduction occurs because of the uptake of external DNA-damaging colicin through its natural receptor by showing that autoinduction was impeded for colicins E2 and E8 in a strain background where the BtuB receptor of these colicins was not functional (Figure S5, Table S3).

Our study of autoinduction in structured environments differentiates between two distinct LexA-based forms of pre-emptive attack for colicins with DNase activity. The E7-like behavior relies mainly on spontaneous SOS response while the E2/E8-like behavior amplifies this basal response through a positive feedback loop whereby previously released colicin forces some of the exposed clonemates to produce toxin themselves. With the former

behavior, toxin production occurs at a lower level through cells dispersed over the entire surface of the colony. With the latter, localized patches of the structured population commit to toxin production, which leads to effective increase of the overall level of released toxin in a cell-density-dependent manner.

Minimal regulatory mutations generate diversity in pre-emptive attack

We found that naturally-occurring colicin operons have different basal expression frequencies (for example E8 versus E2, Figure 1A) and show different autoinduction levels (for example E7 versus E8, Figure 2A). The evolution of natural diversity in aggressive strategies is predicted by evolutionary game theory [4, 7] and has been observed across diverse animal species [1]. Our system gives us an opportunity to study the molecular basis of the variability in aggressive behaviors in bacteria.

Based on the known biology of colicins [22, 34], we hypothesized that the variation in affinity for LexA among colicin promoters could have a major impact on the frequency of colicin-producing cells and, therefore, on the intensity of pre-emptive attacks. To test this, we introduced naturally-occurring point mutations to the LexA-boxes in the colicin E2 promoter to alter their predicted binding affinity for LexA (according to their HI). We designed two variants, one containing higher-affinity LexA boxes and one containing lower-affinity LexA boxes compared to the wild-type promoter. The higher-affinity LexA motif resulted in lower frequency of spontaneous colicin producers while the lower-affinity LexA boxes had the opposite effect (Figure 3AB). In accordance to this result, a strain harboring a modified colicin E2 plasmid where the natural promoter was replaced by the high-affinity one, was less toxic to a nearby competitor that the wild-type colicin E2 strain (Figure S6).

Could autoinduction be controlled as easily? We reasoned that differences in the intracellular concentration of the immunity protein could account for different autoinduction levels (Figure S5C). To test this hypothesis, we created a BZB1011 strain that constitutively overexpresses the E2 immunity protein (Table S3). While this genotypic modification did not affect the basal level of colicin E2 production, it abolished autoinduction in colonies containing both the reporter and the colicin E2 plasmid (Figure 3CD). As previously proposed [48, 49], a higher basal expression level of the immunity protein could also explain the absence of autoinduction from the natural colicin E7 plasmid. This means that in nature autoinduction could be easily modulated by point mutations on the promoter of the immunity protein.

Overall, these results suggest that mutation can easily create a wide range of pre-emptive combat strategies in *E. coli*, by fine-tuning independently either the basal frequency of the attacking cells (through mutations on the promoter of the toxin) or the level of autoinduction in a strain (through mutations on the promoter of the immunity protein).

Competition sensing drives responsive attacks

We next studied the effects of direct competition on bacterial aggression by growing colonies harboring colicin E2, E8 or A reporter plasmids next to different competitor strains. All three reporter strains showed a strong fluorescent signal, localized at the interface of the two colonies, when they were grown next to a strain producing a DNase colicin (E2 or E8), but not next to a non-producing strain (Figure 4A). This behavioral response is consistent with LexA cleavage after induction of SOS response due to the action of DNase colicins. We confirmed this by showing no fluorescent signal was observed when the same experiment was repeated with a BZB1011 strain bearing an uncleavable *lexA* allele (Figure 4B). However, unlike the cases of pre-emptive attack described previously, in this case LexA regulation generates a responsive attack through competition sensing [10]. Colicinogenic

strains sense a competitor and upregulate toxin production as a direct result of the DNA damage this competitor inflicts.

When we repeated our experiments using cells harboring the E2, E8 and A reporter fusions along with the corresponding colicin plasmids (Figure 4C), we observed an upregulation of toxin production for colicins E2 and E8, but not A. Increased toxin production due to autoinduction could already be seen when our focal E2 and E8 strains were placed next to a non-producer strain (Figure 4C, top row). However, a much greater increase in activity over most of the focal colony was seen in the presence of another DNase-colicin producer (Figure 4C, bottom row). This indicates that there is a strong interaction between the autoinduction and the competition sensing colicin production modes. Specifically, the cells closest to the interface with the competitor, via their own response and colicin release, seem to be informing the cells further away from the competitor of the incoming attack. The ability to share information on incoming attacks is well known in animals, where it occurs via alarm calling and alarm pheromones which are used to communicate a perceived threat [50]. The intriguing possibility that social bacteria are capable of similar collective behaviors led us to examine this further.

Competition sensing and autoinduction drive coordinated attacks

A time series of the competition between two autoinducing strains (E2 and E8) revealed a wave of activation that started at the interface between the two strains and moved towards the other edge of the colonies (Figure 5A, Video S2). It is possible that this effect is driven purely by escalating cross-induction between the two DNA-damaging colicins (competition sensing) and that autoinduction does not play a role, which would greatly weaken the analogy to alarm calling in animals. To examine the possibility that cross-induction alone explains toxin production, we paired an autoinducing strain (the wild-type colicin E2 producer) with an E8 producer that is not capable of competition sensing or autoinduction. For the E8 strain, we used a *btuB*-negative colicin E8 producer, which, being unaffected by the toxin of its competitor or its own, produces a constant amount of its own colicin (Figure 5B, top six panels, Video S3). While the *btuB*-negative E8 strain was not capable of competition sensing, the E2 producer developed a strong response at the interface with its competitor, which quickly swept over the entire colony. This result is consistent with autoinduction driving the colony's response rather than cross-induction between the two strains. However, to definitively show that autoinduction is important, we also studied our colicin E2 producer derivative that is capable of competition sensing but incapable of autoinduction (*i.e.* the colicin E2 producer that overexpresses the E2 immunity protein, see Figure 3CD), in the same experimental setting. In this case, competition sensing occurred at the interface with the E8 producing strain, but the signal did not expand further into the colony as communication of the threat was prevented (Figure 5B, bottom six panels, Video S3).

We have, therefore, shown that the combination of a responsive attack and autoinduction drives a coordinated attack. But is this phenomenon really an evolved strategy comparable to those that have been well characterized in animals? One necessary condition for observed phenotypes to represent evolved strategies is the potential for variability on which natural selection can act. The ability to modulate the responsiveness of colicin production via changes on the promoter of the immunity protein is consistent with this being a readily evolvable trait (Figure 3CD). Moreover, the observed natural variation in the propensity of colicin producers for autoinduction (compare E7 with E2 and E8, Figure 2AB) suggests that coordinated-attack behavior is beneficial under some conditions but not others in nature.

A second condition for the coordinated attack to represent an evolved strategy is that it provides evolutionary benefits. A key feature of the collective response is its intensity;

colicin E2 and E8 strains produce a high level of toxin. Game theory and previous experimental work suggests that investing in toxins should be most beneficial when a strain is competitively dominant. The effects of dominance could occur either because a strain possesses a particularly effective weapon or because the contests that are important for fitness are those where it is in the majority, i.e. defending an established colony with colicins is more important for fitness than using colicins to invade a new patch [16, 51]. Based on this prediction, we studied the benefits of coordinated attacks in scenarios where our focal strain is in the majority or in the minority. Specifically, we placed our focal E2 strain (autoinducing/wild-type or non-autoinducing) next to a competing colony which produces colicin E8. When the colicin E2 producer is the majority player, autoinduction provides a competitive benefit (Figure 6, exclusion scenario). By contrast, when it is in the minority, there is no significant difference between the two E2 strains, with the autoinducing strain doing slightly worse (Figure 6, invasion scenario). Therefore, as predicted by theory, we find that dominant strains can benefit from using an intense and highly responsive attack. This is consistent with an evolved strategy used by some strains to rapidly eliminate weaker competitors.

DISCUSSION

We have used colicinogenic plasmids as a model system to study the diversity in antagonistic behavior in bacteria. We chose colicins whose production is under the control of the transcriptional repressor LexA, because this form of regulation is widespread for diffusible bacterial toxins. Despite the simplicity of its design, LexA-based regulation brings about a surprisingly broad spectrum of aggressive behaviors. These occur through variability in two key modes of colicin production, the spontaneous mode and the competition-sensing mode.

We found that several different colicins are produced spontaneously at concentrations that are sufficient to inhibit the growth of nearby sensitive competitors (Figure S1B). Moreover, our data suggest that this spontaneous production is not a regulatory accident but an evolved strategy of pre-emptive attack. The SOS boxes located in the colicin promoters are consistently predicted to have low affinity for LexA and can, thus, readily respond to natural variations in the intracellular concentration of the regulator (File S1 - Tab 1). In agreement with this, we find that toxin production occurs in a higher fraction of the cells than that experiencing stringent SOS response (Figure S2BC). We also showed that the cells earmarked for spontaneous colicin production (and subsequent lysis) have lower reproductive value and are less likely to leave descendants than the average cell in a colicinogenic strain (Figure 1C). In this way, the cost of spontaneous colicin production is minimized but, due to the looser transcriptional repression of the toxin, the colicinogenic strain still mounts a constant and efficient pre-emptive attack.

The intensity and spatial distribution of pre-emptive attacks can be easily modulated by natural selection creating a whole range of basal toxin expression frequencies corresponding to variations in aggressiveness adapted to different ecological contexts. This can be achieved via single nucleotide variations in the LexA boxes of the colicin promoters (Figure 3AB) or the intracellular concentrations of the immunity protein (Figure 3CD) that offers the potential for autoinduction (Figure 2, S3-S5).

The most striking behavior we see, however, stems from the competition-sensing mode of colicin production (Figure 4). The presence of DNA-damaging agents originating from competing strains, triggers LexA cleavage, global SOS response and elevated toxin production. When combined with autoinduction, we found that this generates a coordinated collective response to incoming attacks (Figure 5). Complex behaviors that arise from the interaction of many individuals are well-known from animals [29, 30]. They are also known

from bacteria who use quorum sensing to regulate traits including luminescence, iron scavenging, biofilm formation and pathogenicity [52]. However, to our knowledge, the generation of a collective response to incoming attacks has not previously been documented in bacteria. The clearest analogy is from social animals that use alarm calls or pheromones to communicate a threat and coordinate attacks [50]. For example, upon detecting a threat to the colony, suicidal attacking honeybee workers release a cocktail of pheromones that increases aggression in sister workers, leading to a collective attack [6].

Colicin E2 and E8 producers achieve their collective behavior through the combination of two LexA-based processes, competition sensing and autoinduction (Figure 6C). When an autoinducing strain senses an incoming attack (DNA damage from a competitor), it engages in a responsive attack against its competitor, by locally releasing toxin molecules, which then act as stress cue among clonemates. The clonemates, even if they were initially unaffected by the competitor, upregulate their colicin production and release toxins through autoinduction (Video S2, S3). The result is a rapid and massive counter-attack that can eliminate an invading competitor (Figure 6). This behavior is most likely restricted to colicins regulated by SOS response, since, for it to occur, a cell has to be able to upregulate its toxin in response to the toxin itself (positive feedback loop). It is unlikely that other colicin regulators have this property; for example, the activity of the second most common transcriptional regulator of colicin genes (Figure S1A, File S1 - Tab 1), FUR, which depends on iron availability, should not be directly upregulated by damage inflicted by DNase, RNase or pore-forming toxins. On the other hand, cross-autoinduction between two LexA-regulated colicins present within the same producing strain (on the same or different plasmids), could occur if one of them has DNA-damaging activity. However, this scenario appears rare; only 21 out of the 4,017 *E. coli* genomes listed in the NCBI database were found to contain a colicin gene with DNase activity along with at least one other SOS-regulated colicin gene (File S1 - Tab 2).

We showed that autoinduction provides a benefit to a focal strain when it has to defend an established colony against outnumbered invaders, but not when the positions are reversed. Although the experimental setup we used does not exactly reflect *E. coli* natural growth conditions, our conclusions may reasonably apply to natural settings, especially to spatially structured communities like biofilms [53]. Facing an invading competitor is a common ecological scenario, which has a strong impact on bacterial fitness [54]. The capacity for resident strains to withstand invasion brings major advantages, and many microbial toxins, including contact dependent toxins like the type VI secretion system, may be required to ensure colony or biofilm cohesion rather than to achieve colonization [55].

In summary, we have shown that, when combined with collective behavior, a two-component regulatory system can generate a wide range of aggressive behaviors in bacteria (Figure 7). More specifically, it can single handedly: 1) generate pre-emptive attacks of variable intensities; 2) preferentially identify cells of low fitness for lysis; 3) generate variability in attack behavior via density-dependent amplification of toxin production; 4) orchestrate coordinated and responsive attacks by combining autoinduction with competition sensing. These coordinated strategies mirror social behaviors like alarm calling in animals but, rather than having an independent alarm signal, the colicin toxin doubles as both the means of attack and communication. This dual function enables the bacterium to generate complex behavior with minimal molecular complexity. Given how frequently toxins are found in microbial genomes, our work raises the possibility that many species use diverse and complex aggressive strategies to overcome competitors.

AUTHOR CONTRIBUTIONS

D.A.I.M, D.G., S.A.W. and K.R.F. designed research; D.A.I.M and D.G. performed research; D.A.I.M and D.G. analyzed the data; D.A.I.M, D.G., S.A.W. and K.R.F. wrote the paper; W.K. provided advice during the preliminary stages of the study.

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DATA AVAILABILITY STATEMENT

All data will be available upon request.

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FIGURES

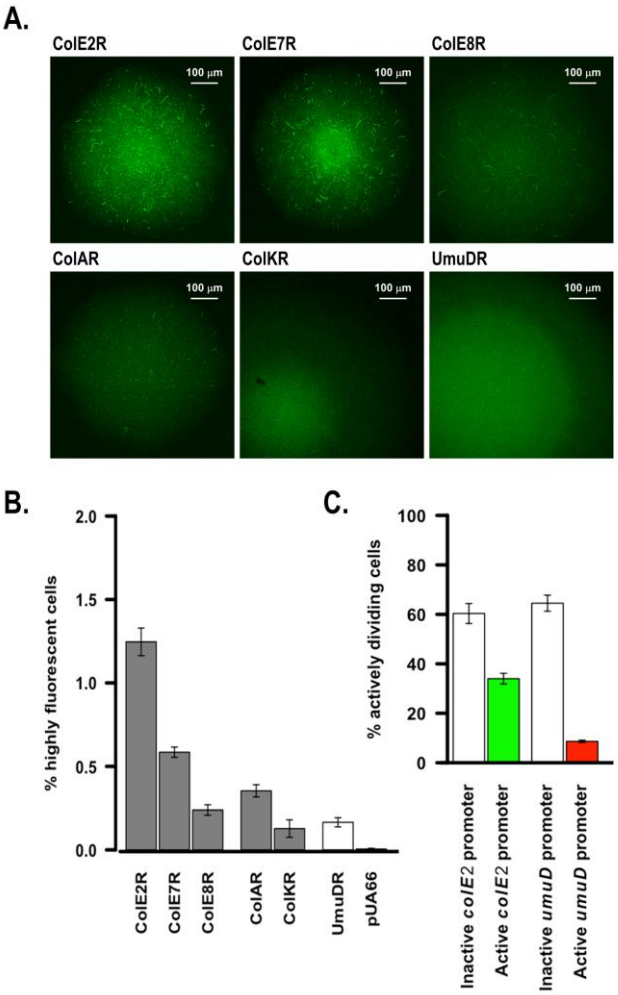

Figure 1. Spontaneous colicin production in colicinogenic strains is heterogeneous and is carried out by weaker cells. A) Single-cell colonies were imaged by confocal microscopy. The promoter of colicins E2, E7, E8, A and K and of the chromosomal gene *umuD* drives the transcription of GFP on a reporter plasmid (labeled ColE2R, ColE7R, ColE8R, ColAR, ColKR and UmuDR, respectively). Induction of the *umuD* promoter is representative of sustained SOS response. B) Frequencies of highly fluorescent cells (cells expressing colicins spontaneously) were quantified by flow cytometry in single-cell colonies of BZB1011 cells harboring colicin reporters (dark grey) or control plasmids (*umuD* reporter or pUA66 vector) (white). The labeling of the reporter plasmids is the same as in (A). The pairwise differences in the average frequency of highly fluorescent cells were significant for all reporter strains according to an ANalysis Of VAriance (ANOVA) and Tukey's HSD test (p-value < 0.05) with the exception of ColE8R, ColKR, and UmuDR strains. Error bars represent the standard deviation of four biological replicates. C) The growth of BZB1011 cells harboring the colicin E2 or the *umuD* reporter plasmids was monitored using fluorescence microscopy for two hours. The frequency of actively dividing cells is shown for cells with active or inactive colicin E2 or *umuD* promoters. The pairwise differences in means were significant based on

596 the Welch Two-Sided Two Sample t-test (p-value < 0.05). Error bars represent the standard
597 deviation of three time courses for each strain.

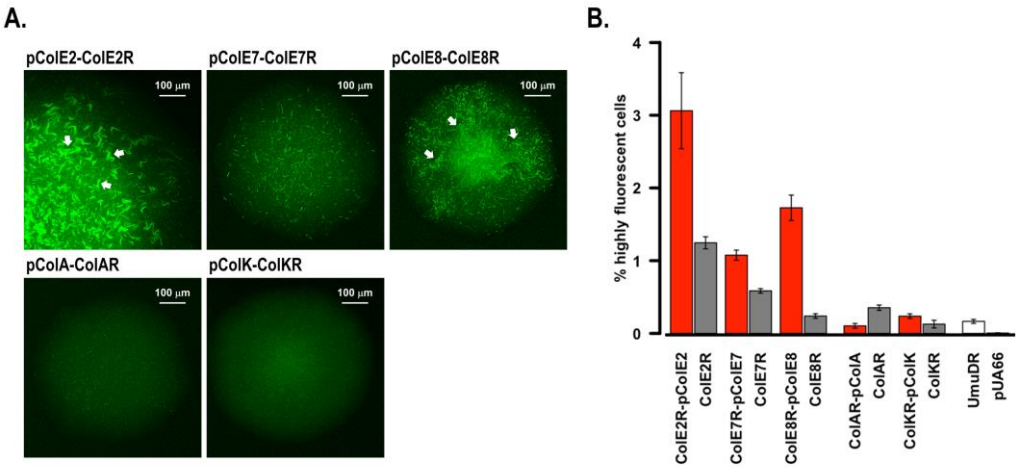

Figure 2. Autoinduction (amplification of spontaneous colicin production through a positive feedback loop) is characteristic of some DNase colicins. A) Single-cell colonies were imaged by confocal microscopy. The promoter of colicins E2, E7, E8, A and K drives the transcription of GFP on a reporter plasmid (labeled ColE2R, ColE7R, ColE8R, ColAR and ColKR, respectively) in cells that also harbor the corresponding colicin plasmids (labeled pColE2, pColE7, pColE8, pColA and pColK, respectively). White arrows indicate patches of autoinducing cells in colonies containing natural plasmids pColE2 and pColE8. Only individual highly fluorescent cells or very small groups (maximum 2-3 cells) can be observed in the colony containing pColE7, suggesting that autoinduction is minimal for this colicin. B) Frequencies of highly fluorescent cells were quantified by flow cytometry in single-cell colonies of BZB1011 strains harboring both colicin and reporter plasmids (red), reporter plasmids only (dark grey) and controls (*umuD* reporter or pUA66 vector only) (white). The labeling of reporter and natural plasmids is the same as in (A). Frequencies of highly fluorescent cells are significantly larger (p-value < 0.05) in the presence of colicin plasmids for E2, E7, E8 and K (Welch One-Sided Two Sample t-tests). Error bars represent the standard deviation of four biological replicates.

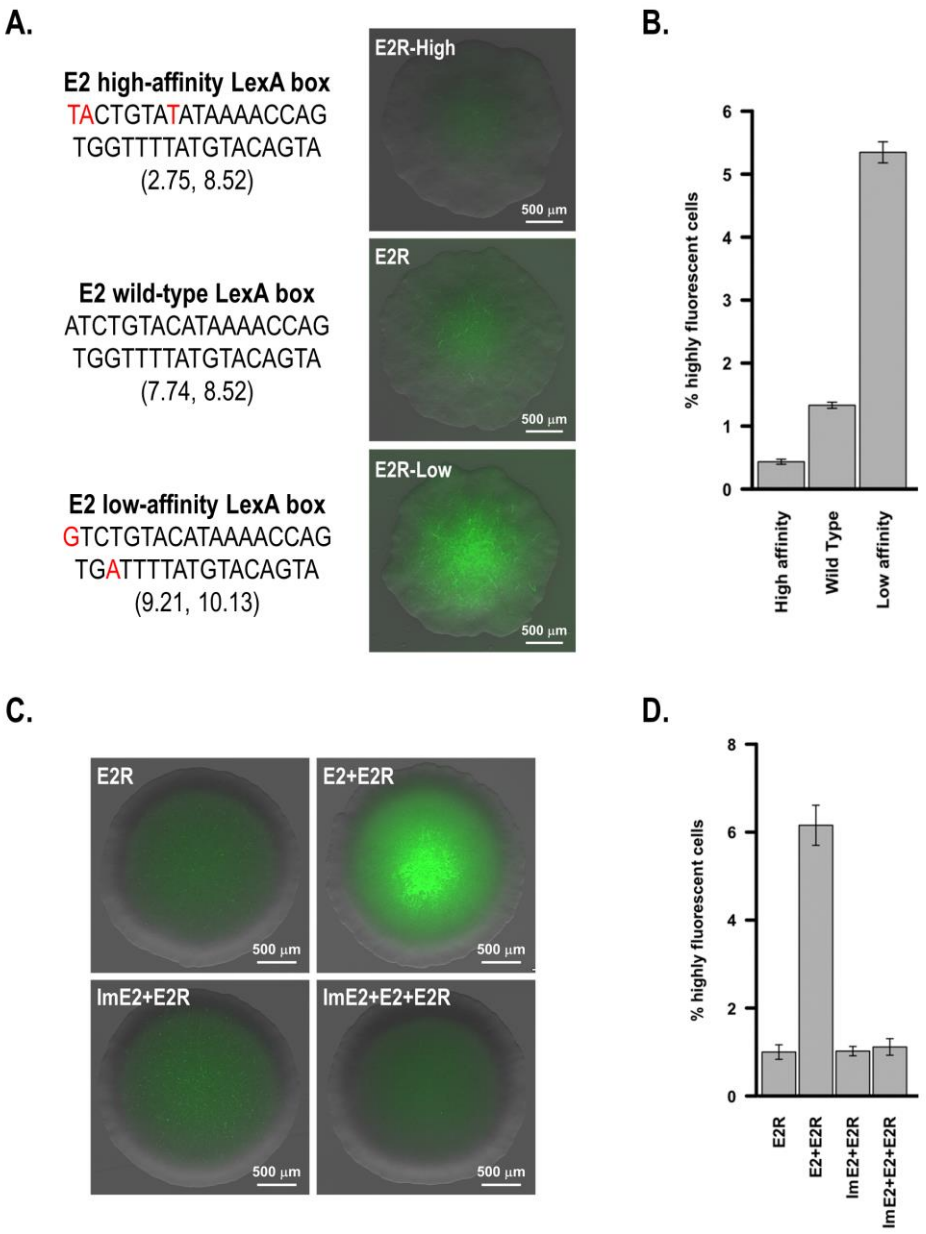

Figure 3. Levels of spontaneous colicin production are readily modified in two ways: by modifying the DNA sequence of the LexA boxes in the colicin promoter or by altering the intracellular levels of cognate immunity protein. A) Single-cell colonies of BZB1011 cells harboring reporter plasmids with colicin E2 promoters designed to have different affinities for LexA (high-affinity variant, labeled E2R-High, top; wild type, labeled E2R middle; low-affinity variant, labeled E2R-Low, bottom) were visualized using a stereomicroscope. The modifications to the wild-type sequences of the LexA boxes are shown in red. The Heterology Index (HI) of LexA boxes (two numbers shown under each DNA sequence) correlates with the frequency of cells that express colicin E2; the lower the HI, the higher the predicted affinity of LexA for the promoter. B) Frequencies of highly fluorescent cells were quantified by flow cytometry in single-cell colonies of BZB1011 strains harboring the three reporter plasmids shown in (A). The pairwise differences among the three strains were all significant based on an ANOVA and Tukey's HSD test (p-value < 0.05). C) Single-cell colonies of BZB1011 cells harboring the colicin E2 reporter were imaged using a stereomicroscope. Images of reporter (E2R) only cells in the presence or

absence of constitutively expressed E2 immunity protein (ImE2) were compared to cells also harboring the colicin E2 plasmid (E2). Overexpressing ImE2 in the presence of the colicin E2 plasmid abolishes autoinduction. D) Frequencies of highly fluorescent cells were quantified by flow cytometry in single-cell colonies of the same strains as in (C). The average frequency of highly fluorescent cells in E2+ER was significantly larger than in the three other strains based on an ANOVA and Tukey's HSD test (p-value < 0.05). For (B) and (D) error bars represent the standard deviation of four biological replicates.

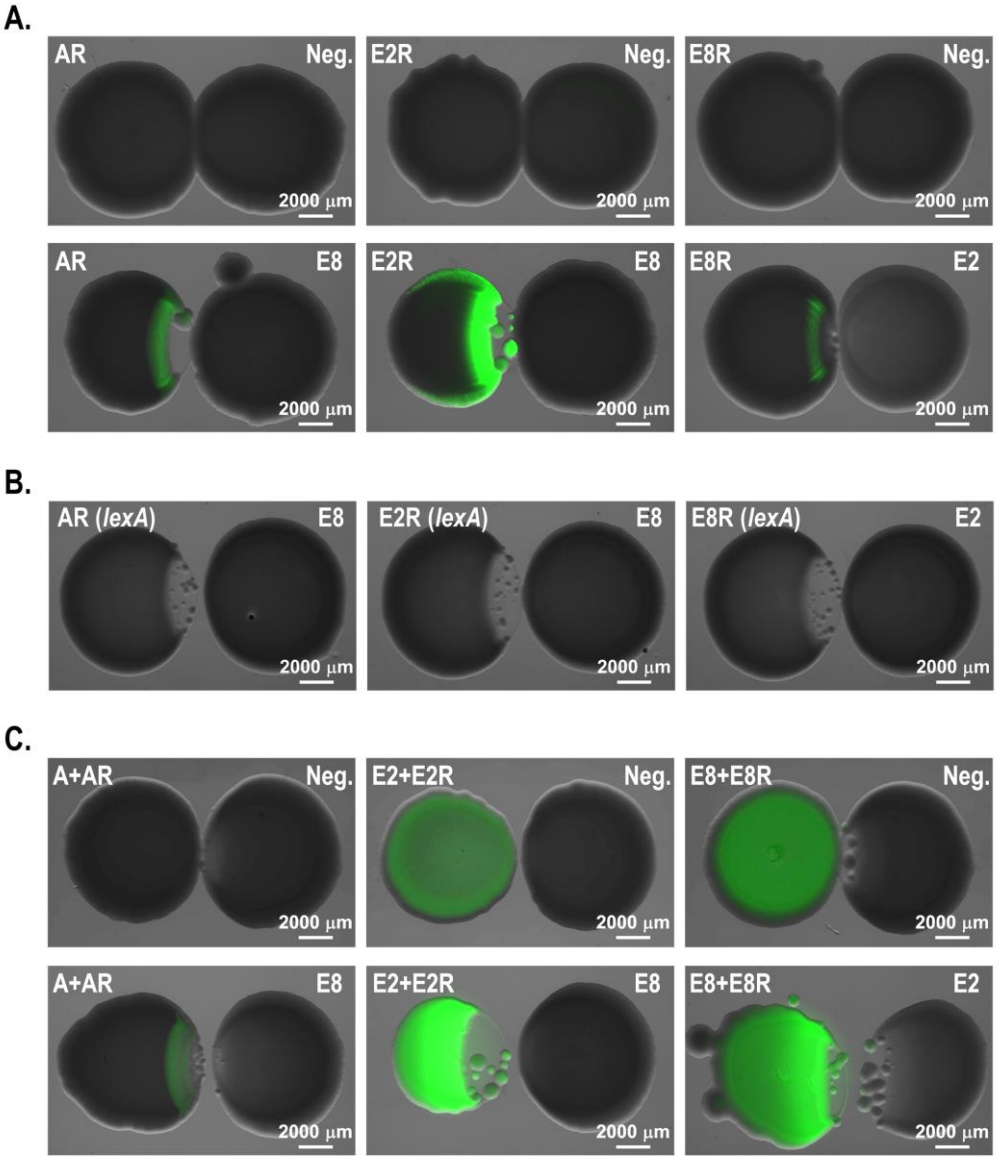

Figure 4. LexA regulation of colicin expression acts as a competition-sensing mechanism, upregulating colicin production when DNA damage is detected. A) BZB1011 cells harboring reporter plasmids, where the promoter of colicins A (left), E2 (middle) and E8 (right) drives the production of GFP, were spotted on the left of each panel. Plain BZB1011 cells (top row) or colicin E8 or E2 producing cells (bottom row) were spotted on the right of each panel. Cells were grown for 12 hours and then imaged using a stereomicroscope. B) The same experiment as in (A) was performed except that BZB1011 cells bearing a non-cleavable *lexA* allele and harboring reporter plasmids for colicins A (left), E2 (middle) and E8 (right) were spotted on the left of each panel. C) The same experiment as in (A) was performed, except that BZB1011 cells harboring the colicin A (left), E2 (middle) and E8 (right) plasmids as well as the corresponding reporter plasmids were spotted on the left of each panel. For all panels, the reporter plasmid for the promoters of colicins A, E2 and E8 is labeled AR, E2R and E8R, respectively, while the natural colicin plasmid of colicins A, E2 and E8 is labeled A, E2 and E8, respectively.

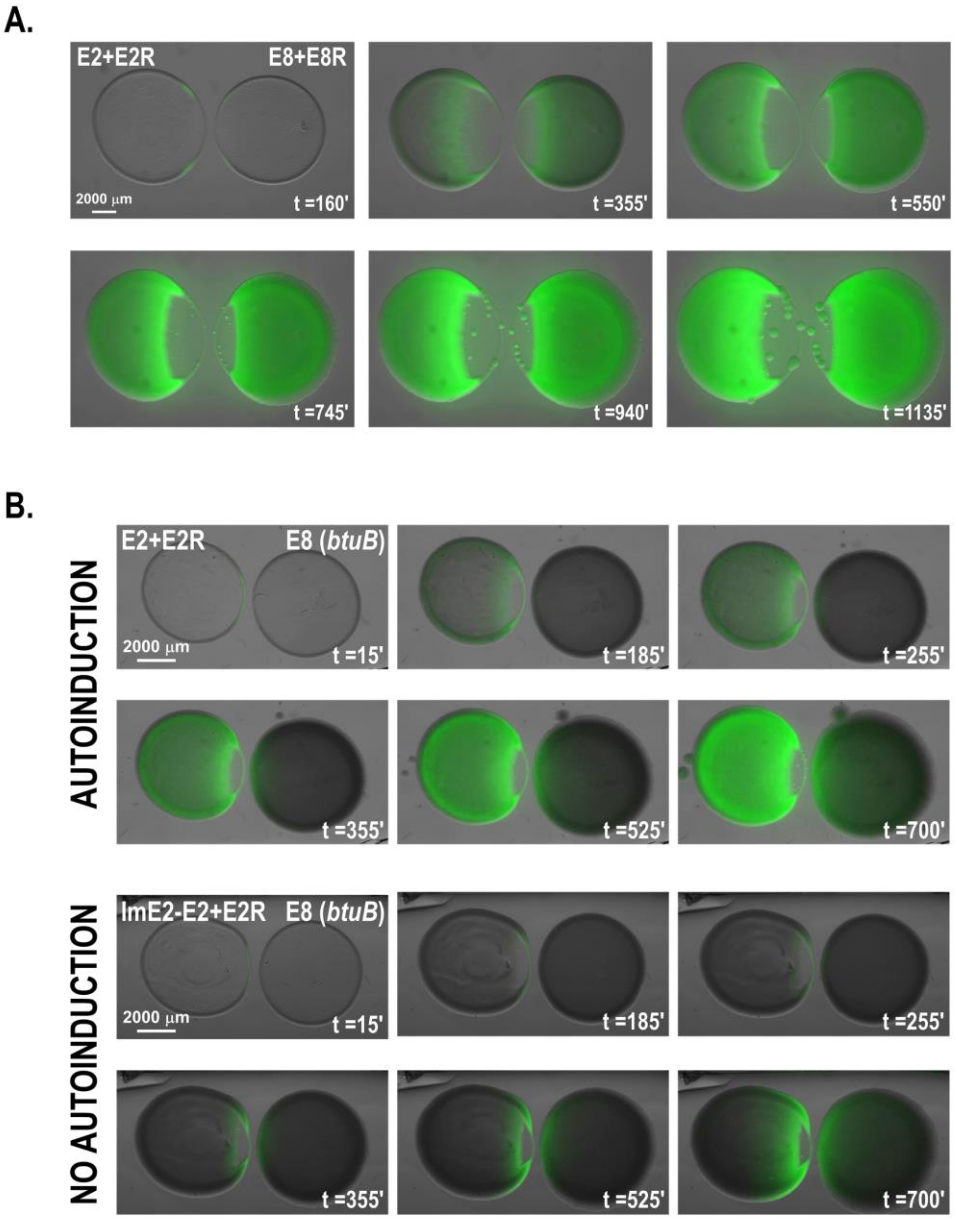

Figure 5. The interaction of colicin upregulation due to competition sensing and autoinduction allows for the emergence of a coordinated attack behavior. A) A time course (Video S2) of the competition between two DNase-producing colicinogenic strains with the ability to autoinduce was recorded using a stereomicroscope and representative snapshots are shown here. BZB1011 cells harboring the colicin E2 and E8 plasmid and the corresponding reporters were spotted on the left and the right, respectively. B) A time course (Video S3) of the competition of two pairs of colicin producers was recorded using a stereomicroscope. Six snapshots are shown for each competition. A *btuB*-negative BZB1011 strain producing colicin E8 was spotted on the right for both competitions. BZB1011 cells harboring the colicin E2 plasmid and the corresponding reporter (autoinducing strain) were spotted on the left, for the top six panels. BZB1011 cells constitutively overexpressing the E2 immunity protein, harboring the colicin E2 plasmid and the corresponding reporter (non-autoinducing strain) were spotted on the left, for the bottom six panels. For all panels, the reporter plasmid for the promoters of colicins E2 and E8 is labeled E2R and E8R,

676 respectively, while the natural colicin plasmid of colicins E2 and E8 is labeled E2 and E8,
677 respectively. Overexpression of the immunity protein of colicin E2 is denoted by ImE2.

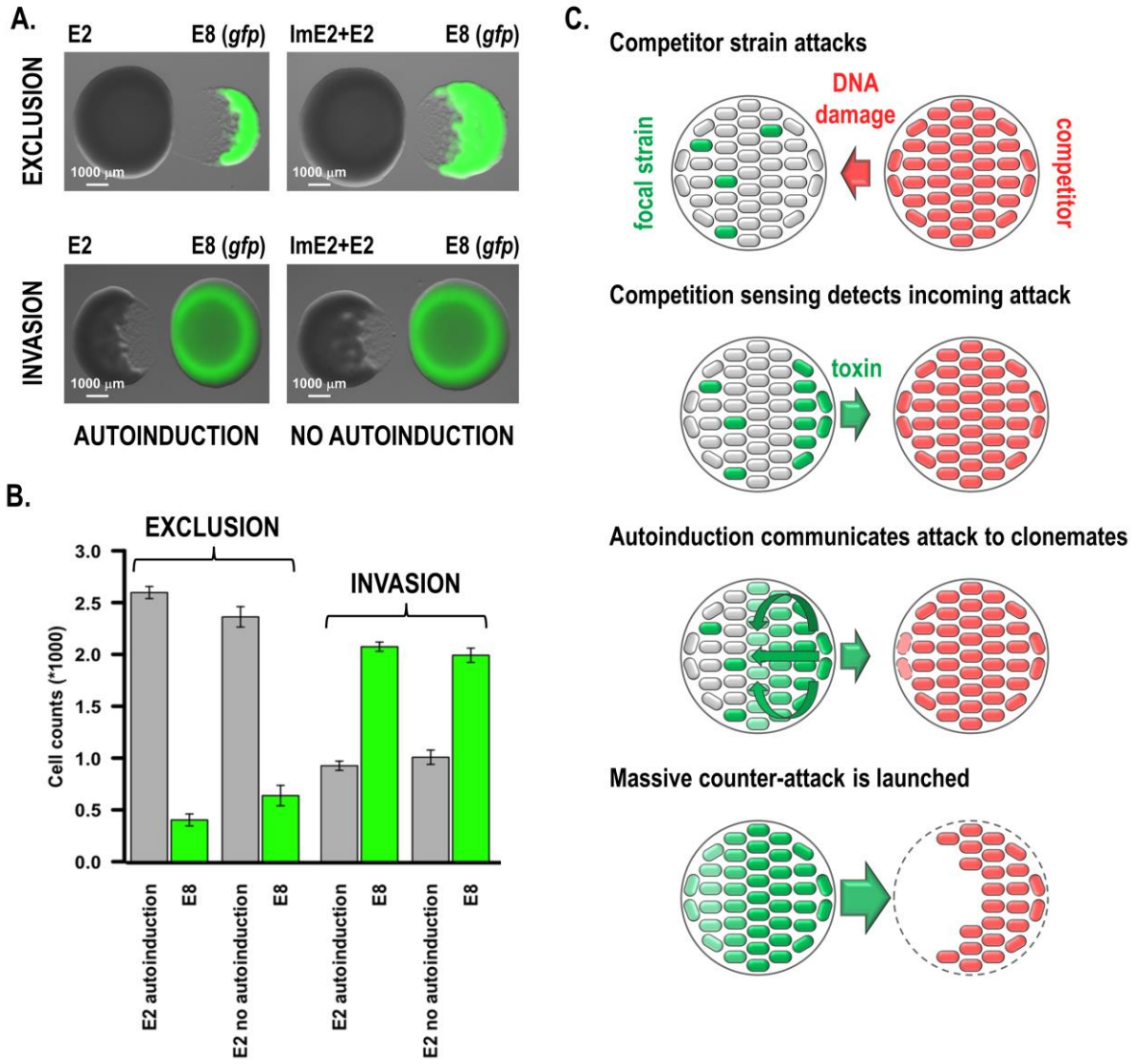

Figure 6. Autoinduction-driven coordination of toxin production provides a benefit to colicinogenic strains in defined conditions. A) BZB1011 cells harboring the colicin E2 plasmid (autoinducing strain, left panels) or BZB1011 cells overexpressing the E2 immunity protein and harboring the colicin E2 plasmid (non-autoinducing strain, right panels) were spotted next to a GFP-labeled BZB1011 strain harboring the colicin E8 plasmid. Cultures of all colicinogenic strains were normalized. Colicin E2 and E8 producers were diluted to 10^{-2} and 10^{-4}, respectively in the top row and to 10^{-4} and 10^{-2}, respectively in the bottom row. The natural colicin plasmid of colicins E2 and E8 is labeled E2 and E8, respectively while overexpression of the immunity protein of colicin E2 is denoted by ImE2. B) Colonies of colicin E2 producers are significantly better at spatially excluding colicin E8 invaders when they can signal for the presence of the competitor (autoinduction), whereas autoinduction does not provide any benefit when colicin E2 producers are spatially invading the territory of colicin E8 producers. An ANOVA confirms the significance of the differences for both the "ecological scenario" and "autoinduction" factors, and detects a strong interaction between the two factors ($p<<0.05$ in all three cases); the difference between autoinducing and non-autoinducing E2 is significant in the exclusion ($p<<0.05$) but not in the invasion scenario ($p>0.99$) in comparisons based on Tukey Honest Significant Difference. Error bars represent

the standard deviation of eight biological replicates. C) Schematic representation of the coordinated attack behavior for autoinducing colicin producers. A focal strain, which is spontaneously producing toxins (left), is attacked by a competitor producing a DNA-damaging toxin (right) (first panel). The focal strain senses the competitor's toxin and cells in the affected part of the colony, at the interface of the two strains, upregulate their colicin production in response (second panel). The released toxin affects the competitor and, at the same time, alerts the unaffected clonemates of the focal strain of the incoming threat (third panel). The rest of the colony upregulates its colicin production through autoinduction enabling the attacked focal strain to launch a massive counter-attack which eliminates the competitor (fourth panel).

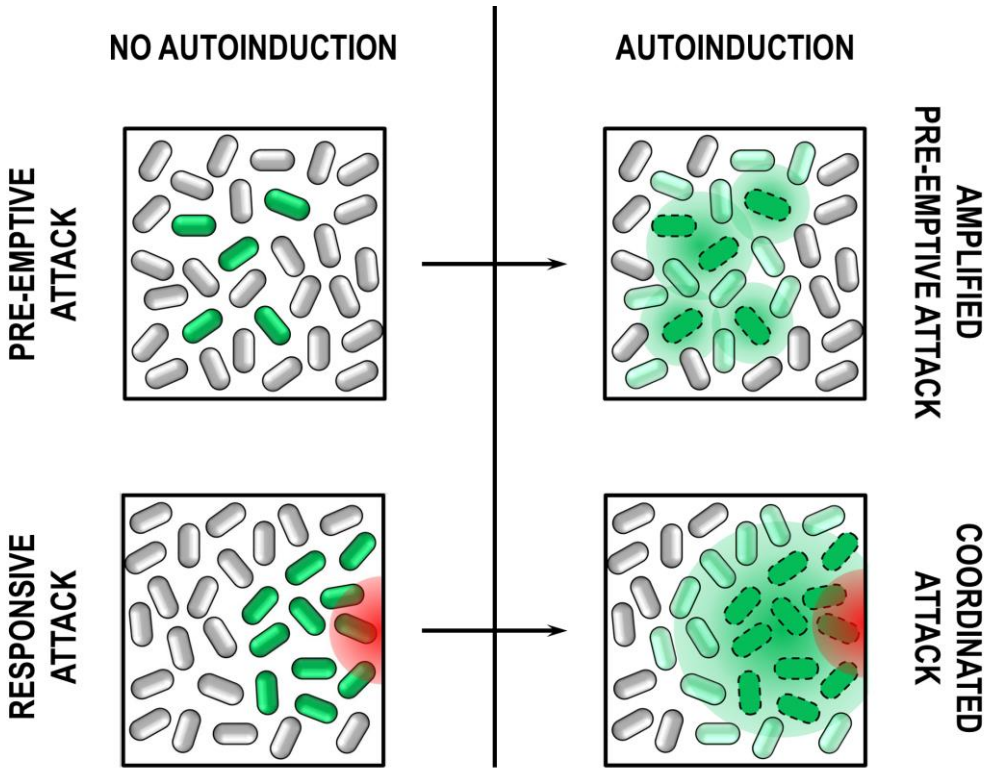

Figure 7. Colicinogenic *E. coli* is capable of diverse and complex aggression strategies. There are two fundamentally different modes of toxin production for LexA-regulated colicins: 1) Colicins are constantly produced at basal levels by a small fraction of the cells that experiences LexA cleavage, lyses and releases colicins generating a pre-emptive attack (top left). 2) Colicin expression is strongly induced by genotoxic agents (red halo) originating from competing strains. In this case, LexA functions as a competition-sensing system and the exposed cells express colicin generating a responsive attack (bottom left). For both modes, if the colicin that is released is a DNase, its production may be locally amplified in a density-dependent manner via autoinduction. For spontaneous colicin production (top right), autoinduction amplifies the intensity of the pre-emptive attack by concentrating the toxin production in specific areas on the colony. In the case of an incoming threat (bottom right), the combination of competition sensing and autoinduction leads to a massive coordinated attack, whereby cells unaffected by the competitor are alerted by their affected clonemates to contribute to the fight.

METHODS

Construction of plasmids. Plasmids and oligonucleotides used in this study are listed in Tables S2 and S4, respectively. DNA manipulations were conducted using standard methods. KOD Hot Start DNA polymerase (Novagen) was used for all PCRs, unless stated otherwise, and all constructs were sequenced and confirmed to be correct before use. Oligonucleotides were synthesized by IDT.

Reporter plasmids were generated using the pUA66 vector as described in [28]. More specifically, for the construction of pUA66-P*colE2*::*gfp*, the *colE2* promoter region (403 bps, from -259 to +144 from the ATG) was amplified from pColE2, using oligonucleotides P1 and P2, and cloned into the XhoI-BamHI sites of pUA66. pUA66-P*colE8*::*gfp* and pUA66-P*umuD*::*gfp* were generated in the same way except that the *colE8* promoter region (444 bps, from -334 to +110 from the ATG) was amplified from pColE8 with primers P3 and P4 and the *umuD* promoter region (480 bps, from -344 to +136 bps from the ATG) was amplified from BZB1011 genomic DNA with primers P5 and P6. For the double reporter pUA66-P*colE2*::*gfp*-P*umuD*::*mrfp1*, a fusion of the promoter region of *umuD* with *mrfp1* was cloned into the XhoI site of pUA66-P*colE2*::*gfp*. The P*umuD*::*mrfp1* insert was amplified using oligonucleotides P5 and P9 from the following two products: 1) the amplification product of primers P5 and P7 on pUA66-P*umuD*::*gfp* and 2) the amplification product of primers P8 and P9 on pUltraRFP-GM.

To generate pGRG25-P*max*::*gfp,* the P*max::gfp* fragment was amplified from pUltraGFP-GM using oligonucleotides P10 and P11, digested with PvuI and NotI and ligated into PacI-NotI-digested pGRG25. To generate pGRG25-P*max*::*immE2,* two overlapping fragments, containing the P*max* promoter and the associated transcriptional terminator bridged by a newly designed multiple cloning site (MCS), were amplified from pUltraGFP-GM using oligonucleotides P14/P15 and P12/P13, respectively. These fragments were annealed and co-amplified during a second PCR step, using oligonucleotides P13 and P14. The resulting DNA fragment was digested with PvuI and MluI and ligated into PacI-AscI-digested pGRG25. The E2 immunity gene was amplified from pColE2 using oligonucleotides P16 and P17, digested with NotI and AscI and cloned into the NotI-AscI sites of the new MCS of the modified pGRG25 plasmid. Both pGRG25-P*max*::*gfp*, and pGRG25-P*max*::*immE2* were used to generate the BZB1011 (*gfp*) and BZB1011 (*immE2*) strains as described below.

To generate a chloramphenicol resistant pColE2 plasmid (pColE2-Cm), the PstI-digested chloramphenicol resistance cassette from pSUP404.2 was blunted using T4 DNA polymerase (New England Biolabs), according to the manufacturer's protocol, A-tailed with OneTaq (New England Biolabs) for 10 minutes at 72°C and cloned into pGEM-T-easy (Promega). The cassette was extracted using NotI and cloned into the NotI-digested amplification product of oligonucleotides P18 and P19 on pColE2 (whole plasmid amplification using Phusion polymerase in HF buffer according to the New England Biolabs' protocol). The E2 colicin operon was sequenced and found to be free of mutations.

To generate the pUA66-P*colE2*-H and pUA66-P*colE2*-L reporter plasmids, oligonucleotides P20 and P21 (for pUA66-P*colE2*-H), and P22 and P23 (for pUA66-P*colE2*-L) were resuspended in molecular biology H_2O at a final concentration of 10µM and annealed by slowly cooling down from 100°C to 25°C. This generated two different 89 bps double stranded DNA fragments, homologous to part of the ColE2 promoter (fragments H and L, respectively). A third fragment (pUA66-P*colE2* fragment) encompassing the whole pUA66-

P*colE2*::*gfp* plasmid except for part of the *colE2* promoter containing the LexA boxes, was amplified with oligonucleotides P24 and P25 using Phusion polymerase in HF buffer according to the New England Biolabs' protocol. Fragments H or L were added to fragment pUA66-P*colE2* using the Gibson assembly Master Mix (New England Biolabs) according to the manufacturer's protocol, to generate plasmids pUA66-P*colE2*-H or pUA66-P*colE2*-L, respectively. Plasmid pColE2-Cm-H was generated in the same way by assembling fragment H and the amplification product of P24 and P25 onto pColE2-Cm. The *colE2* promoter sequences in all three plasmids as well as the E2 colicin operon in pColE2-Cm-H were sequenced and found to be free of mutations.

Construction of bacterial strains. Bacterial strains used in this study are listed in Table S3. The *lexAind3* mutation was transduced from strain JJC443 [56] into BZB1011 using P1 phages according to the protocol described in [57]. Transductants were selected on 10 µg ml^{-1} tetracyclin and the presence of the *lexAind3* mutation was confirmed by DNA sequencing. The *btuB* mutation was generated by growing cells next to an E8 colicinogenic strain on 1.5% w/v LB agar medium. *De novo* resistant colonies were isolated, their *btuB* gene was amplified, and the amplification product was sequenced. In all cases, the *btuB* gene was found to be disrupted by an endogenous transposon.

For the generation of the BZB1011 (*gfp*) strain, BZB1011 cells were transformed with pGRG25-P*max*::*gfp* and selected on 100 µg ml^{-1} ampicillin at 30°C. The resulting strain was cultured for 12 hours in LB medium (10 g l^{-1} peptone, 5 g l^{-1} yeast extract, 10 g l^{-1} NaCl) at 30°C in the absence of ampicillin and in the presence of 0.5% w/v arabinose. 5µl of this culture were streaked onto 1.5% w/v LB agar medium containing 0.5% w/v arabinose and incubated at 42°C for 12 hours Fluorescent colonies were selected, streaked onto 1.5% w/v LB agar medium and incubated at 42°C for 12 hours. Resulting colonies were tested for ampicillin sensitivity and for the presence of the P*max*::*gfp* fragment. The pColE8 natural plasmid was introduced into this strain using electroporation and selected on 1.5% w/v LB agar medium containing 50% v/v filter-sterilized spent medium obtained from an overnight culture of an E8 colicinogenic strain. The resulting strain was tested for production of colicin E8 using killing assays.

For the generation of BZB1011 (*immE2*), BZB1011 cells were transformed with pGRG25-P*max*::*immE2* and selected on 100 µg ml^{-1} ampicillin at 30°C. The resulting strain was cultured for 12 hours in LB medium at 30°C in the absence of ampicillin and in the presence of 0.5% w/v arabinose. 5µl of this culture were streaked onto on 1.5% w/v LB agar medium containing 0.5% w/v arabinose and 50% v/v filter-sterilized spent medium obtained from an overnight culture of an E2 colicinogenic strain, and incubated at 42°C for 12 hours. Resulting colonies were streaked onto 1.5% w/v LB agar medium and incubated at 42°C for 12 hours. Colonies were tested for ampicillin sensitivity, presence of the P*max*::*immE2* fragment, and resistance to colicin E2 (using killing assays).

All reporter plasmids were introduced in the relevant strains using electroporation and selected on kanamycin at a final concentration of 50 µg ml^{-1}. BZB1011 cells already harboring the natural colicin plasmids were used for all experiments described in this study with the exception of the experiments described in Figures 3CD and S6. In this case pColE2-Cm and pColE2-Cm-H were introduced into the relevant strains by electroporation and selected on 1.5% w/v LB agar medium containing chloramphenicol at a final concentration of 10 µg ml^{-1}.

Bacterial growth conditions. All liquid cultures were grown in 5 ml LB medium in 50 ml polypropylene tubes; they were incubated for 12 hours at 37°C with shaking at 250 rpm. For the generation of single-cell colonies, 20 µl of a 10^6-fold dilution of an overnight culture were platted on 1.5% w/v LB agar medium and incubated for 12 hours at 37°C. When needed, kanamycin or gentamycin were added to the media at 50 µg ml^{-1} and 30 µg ml^{-1}, respectively.

For experiments described in Figure S1 and S6, 0.8% w/v LB agar medium was used. The dense colonies on the right-hand side of each panel were generated by spotting 10 µl of an overnight culture whereas the dilute non-producers of the left-hand side of each panel were generated by spotting 5 µl of a 10^6-fold dilution of an overnight culture. Plates were incubated at 37°C but the incubation time varied depending on the colicinogenic populations that were tested. Experiments described in Figure 4 and 6 were also carried out on 0.8% w/v LB agar medium. 10 µl or 5 µl of an overnight culture were spotted for all strains in Figure 4 or 6A, respectively and the plates were incubated for 12 hours at 37°C.

Flow cytometry. 1-4 µl of stationary- or exponential-phase cultures were diluted into 200 µl of filter-sterilized M9 medium without a carbon source (12.8 g l^{-1} $Na_2HPO_4.7H_2O$, 3 g l^{-1} KH_2PO_4, 0.5 g l^{-1} NaCl, 1 g l^{-1} NH_4Cl, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$). Alternatively, small colonies were thoroughly resuspended into 250 µl of filter-sterilized M9 medium and the suspension was 10-fold further diluted in filter-sterilized M9 medium. Spots grown for competition experiments (in Figure 6) were thoroughly resuspended in 1 ml of filter-sterilized M9 medium. 4 µl of this suspension were further diluted into 200 µl filter-sterilized M9 medium. All cell suspensions were analyzed on a BD Accuri C6 Flow Cytometer using 10,000 and 8,000 as thresholds for the FSC and SSC parameters, respectively. When colonies or spots were used, the population of cells was further gated to avoid biases due to clumps. For each strain and condition, at least four biological replicates were analyzed and 20,000 events were quantified per sample.

Flow cytometry analysis. All flow cytometry data were analyzed with R (http://www.R-project.org).

When assessing the expression levels of reporter plasmids in colonies or liquid cultures (in Figures 1-3, S2, S3 and S5), a cell was classified as 'highly fluorescent', if its fluorescence value FL1 (FL) was at least 3 times higher than the median of the population; the cell size (FSC) was also taken into account in the calculation. This relationship is given by the following equation where FL and FSC values are log2 transformed:

$$3 * [cell\ FL] - [cell\ FSC] - 3 * [median\ population\ FL] + [median\ population\ FSC] - 6 > 0$$

When quantifying the abundance of different populations in competition experiments (in Figure 6), only events within the core of the cell population were considered in order to minimize count biases. Practically, the density of the bacterial population was calculated on the FSC and SSC dimensions and only events within the denser part of the population were further processed. The minimal FL threshold value empirically chosen to discriminate cells bearing the Tn7::P*max::gfp* construct was $2^{9.5}$. Counts shown in Figure 6B are based on the first 3000 events recorded that met our selection criteria.

Imaging. Still pictures of colonies (in Figure S1) were generated using an EOS 30D DSLR camera (Canon). Images for Figures 3, 4, 6 and S6 were acquired using a Zeiss PlanApo Z

0.5×objective on an AxioZoom.V16 microscope (Zeiss) with the associated Zen software; entire plates were imaged without disturbing the agar surface. Confocal imaging of single cell-colonies (in Figures 1, 2, S2, S4, S5) was carried out using a Zeiss Plan-Apochromat 10x objective on a Zeiss LSM700 scanning laser confocal unit with the associated Zen software; a square piece of 1.5% w/v LB agar medium containing the entire colony was cut out and placed on slides without a coverslip. For epifluorescent microscopy (in Figure S2), 5 µl of an overnight culture were placed onto a square piece of 1.5% w/v LB agar medium and covered with a n° 1.5 cover slip. Imaging was done using a Zeiss Axio Observer inverted microscope with a Zeiss Plan-Apochromat 63x oil immersion objective (NA=1.4), a Zeiss AxioCam MRm camera, a Zeiss Definite Focus system and the associated Zen software. For each experiment, all microscope images were acquired and processed using identical settings.

To record time courses at a single-cell level (in Figure 1 and Video S1), 5 cm diameter glass bottom Petri dishes with a 3-cm diameter uncoated n° 1.5 glass window (MatTek Corporation) were used. 8 µl of a 2-fold dilution of an overnight culture were placed onto a 2.8 cm diameter (0.5 cm thickness) 1.5% w/v LB agar slab which was, in turn, placed face down on the glass bottom window of the Petri dish so that the cells were sandwiched between the glass and the solid LB medium. Subsequently, the slab was completely encased with 1.5% w/v LB agar and the Petri dish was positioned on a heated stage at 37°C. The Zeiss Axio Observer inverted microscope with a Zeiss Plan-Apochromat 63x oil immersion objective (NA=1.4), a Zeiss AxioCam MRm camera, and a Zeiss Definite Focus system was used and images were acquired every 1 or 2 min., depending on the experiment, for up to two hours. For the data presented in Figure 1C, three biological replicates of each time course were carried out, and for each replicate 16 fields of view were recorded at each timepoint.

To record time courses of competition experiments (in Figure 5 and Videos S2 and S3), 5 µl of each washed and normalized overnight culture were spotted onto 0.8% w/v LB agar medium on 5 cm diameter Petri dishes. The latter were placed face up and were covered by the heated stage used for single-cell time courses; the heating element of the stage was in full contact with the lid of the plate. A large n° 1.5 cover slip was used to cover the viewing window of the stage in order to avoid condensation in the interior of the Petri dish. Images were acquired at 37°C every 5 min for up to 19 hours using the Zeiss PlanApo Z 0.5×objective on the AxioZoom.V16 microscope (Zeiss) with the associated Zen software.

Bioinformatics. The NCBI Plasmid database (last updated July 2015) was searched for homologues of colicins using blastp [58] (evalue < 1e-10, percent identity > 40). The NCBI gi numbers of the querries used are the following: Alveicin A (499491735), Alveicin B (49234738), Cloacin DF13 (10955256), Col10 (807876), Col5 (1212893), ColA (9507292), ColB (727400684), ColD (695196167), ColE1 (9507254), ColE2 (204309820), ColE3 (695196661), ColE4 (754716641), ColE5 (587677783), ColE6 (144395), ColE7 (144375), ColE8 (242347932), ColE9 (222098967), ColFY (410688433), ColIa (695197212), ColIb (9507452), ColK (61965912), ColM (169546453), ColN (748133257), ColR (542675452), ColS4 (5295807), ColU (695198141), ColV (84060908), ColY (10955435), Klebicin B (11342751). To identify the regulators of colicin genes, the 200 bps upstream the start codon (ATG) of each gene (considered the 'promoter region') were analyzed for potential binding sites. The presence of a CRP binding site was inferred if the sequence 'CTGTATAT' was included in the promoter region, with one mismatch authorized. LexA, IscR or FUR binding sites were identified using the heterology index (HI) measure. For this, a positional matrix model was built on the basis of a list of known LexA [59], IscR type 1 [60] and FUR [61] binding sites. HI provides a measure for heterology between an examined sequence and the

925 generated model and it is calculated following the method described in [62]; high HI values
926 mean low affinity of the regulator for the binding site. Maximal thresholds chosen for LexA,
927 IscR and FUR were 14, 30 and 14, respectively.

LEGENDS FOR SUPPLEMENTAL VIDEOS

Video S1 (related to Figure 2). Spatial proximity is crucial for autoinduction. A time course was recorded by fluorescence microscopy using BZB1011 cells harboring the colicin E8 natural plasmid and its reporter plasmid, where the promoter of colicin E8 drives the production of GFP. Propidium iodide was added to the cells; when a cell becomes permeable (lyses) binding of propidium iodide to its DNA displays red fluorescence. Lysis of the only cell producing colicin in the field of view (initially green and subsequently red), leads to induction of colicin expression in its neighboring cell which in turn expresses colicin (marked by GFP production, green) and subsequently lyses (red). The two cells that are flanking the lysing pair elongate and divide irregularly as they are also affected by the released DNase toxin, but do not produce colicin. The remaining cells in the field of view are not affected and divide regularly. The video is presented at 7 frames/sec and the interval between frames is 1 min.

Video S2 (related to Figure 5). Competition sensing and autoinduction drive an escalation in colicin production between two DNAse-producing strains. A time course of the competition between two DNase-producing colicinogenic strains with the ability to autoinduce was recorded using a stereomicroscope. BZB1011 cells harboring the colicin E2 and E8 plasmid and the corresponding reporters were spotted on the left and the right, respectively. The video is presented at 5 frames/sec and the interval between frames is 5 min.

Video S3 (related to Figure 5). The combination of colicin upregulation due to competition sensing and autoinduction allows for the emergence of a coordinated-attack behavior. A time course of the competition between two pairs of colicin producers was recorded using a stereomicroscope. In order for the two competitions to be comparable, a *btuB*-negative BZB1011 strain producing colicin E8 was used (spotted on the right for both competitions). This strain is immune to the toxin of its competitor and produces a constant amount of its own toxin (basal production without autoinduction or competition sensing). BZB1011 cells harboring the colicin E2 plasmid and the corresponding reporter (autoinducing strain) were spotted on the left, for the top pair. BZB1011 cells constitutively overexpressing the E2 immunity protein and harboring the colicin E2 plasmid and the corresponding reporter (non-autoinducing strain) were spotted on the left, for the bottom pair. The video is presented at 5 frames/sec and the interval between frames is 5 min.

LEGENDS FOR SUPPLEMENTAL FILES

File S1. A) (Tab 1, related to Figure S1) Close homologues of known colicin activity proteins found in the NCBI plasmid database. The presence of IscR, CPR, FUR and LexA binding sites is indicated by green, yellow, pink and purple shading, respectively. Sites with high (score < 20) and lower (score > 20) predicted affinity for IscR are marked with dark green and light green, respectively. The number of binding sites for LexA and their relative score (cutoff of 14) are also indicated. Tandem LexA sites (less than 20 bp apart in sequence) are marked with an asterisk. **B) (Tab 2, related to Figure 2**) ***E. coli* strains capable of cross-autoinduction.** Some colicinogenic strains contain a colicin gene with predicted DNase activity and at least one more LexA-regulated colicin gene. This which exposes them to cross-autoinduction. The genomes of 4,017 *E. coli* strains in the NCBI database were searched for colicin homologs using blastp [58] (evalue < 1e-10, percent identity >70%, coverage >70%). LexA binding sites were predicted on the 200 bps upstream the start codon

of the putative colicin genes as described in the “Methods” section. 21 strains were found to contain a colicin with predicted DNase activity and at least one more LexA-regulated colicin gene.

SUPPLEMENTAL FIGURES

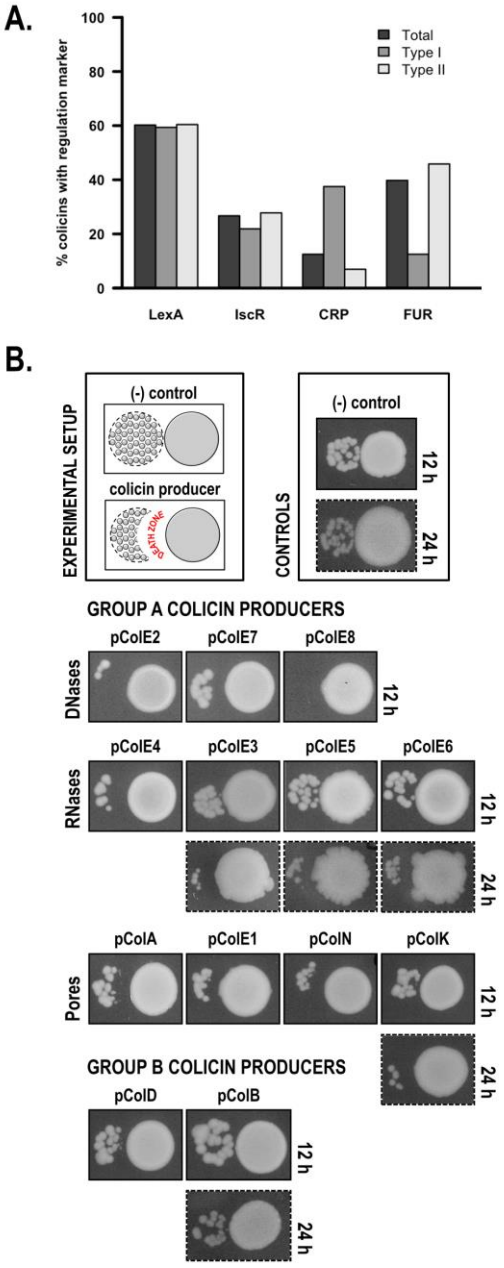

Figure S1 (related to Figure 1). Spontaneous colicin production is widespread and biologically significant. A) The graph shows the frequency of occurrence of binding sites for the SOS repressor LexA, the metabolic stress regulators IscR and CRP, and the iron limitation regulator FUR in the promoter region of genes encoding colicin-like proteins. Type I plasmids are as large as 10 kb and type II plasmids are larger than 10 kb. LexA is the most common regulator of colicin-like toxins in Enterobacteria. It is found to regulate the expression of colicins of any toxicity (i.e. DNases, all types of RNases and pore-forming toxins); in *E. coli* strains DNase colicins are almost always regulated by LexA. FUR is the second most common regulator of colicin-like toxins. It responds to iron availability, which is most likely a proxy for starvation stress, and it regulates mainly pore-forming toxins in small plasmids or

colicins encoded by large (type II) plasmids. B) Spontaneous toxin production driven by LexA is common across colicinogenic strains. On the right of each panel a dense colicinogenic population was grown for 12 (solid line contours) or 24 hours (dotted line contours). A 10^6-fold dilution of an overnight culture of a non-colicinogenic population was grown to the left of each producer for 12 hours. The label on top of each panel refers to the natural colicin plasmid harbored by the dense bacterial population. Control experiments with a non-colicinogenic strain are shown in the right inset; the same strain background was used for all populations. The left inset shows the experimental setup and what is to be expected in the presence or absence of the natural colicin plasmid.

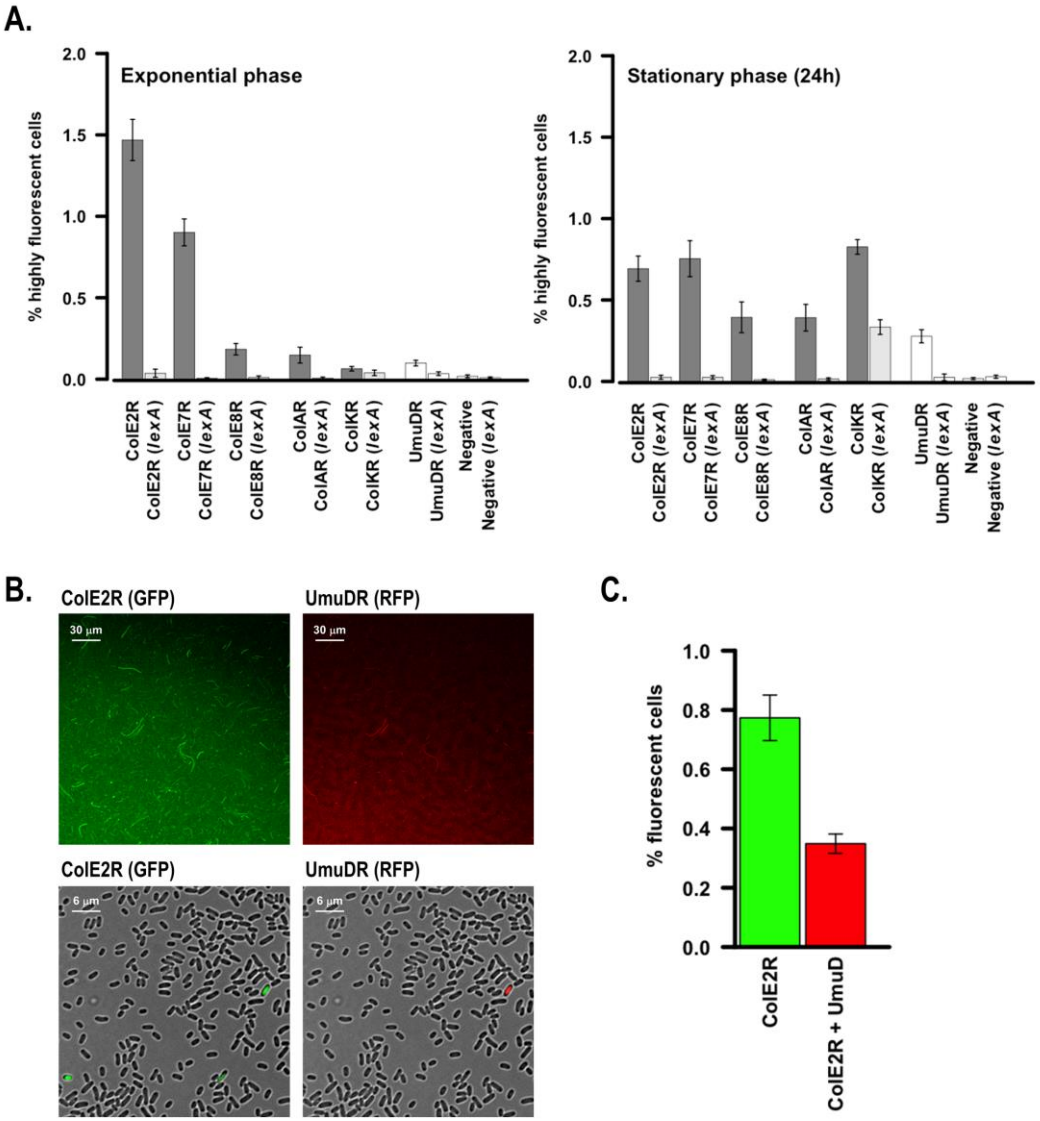

Figure S2 (related to Figure 1). The frequency of spontaneous colicin production is heterogeneous, but it is higher than that of stringent SOS response. A) The frequency of spontaneous colicin expression varies depending on the growth phase. The graphs show the frequency of cells with high GFP expression in liquid culture in exponential phase (OD600 = 0.3) and stationary phase (24-hour culture) in the wild type BZB1011 strain (dark grey) or in an isogenic BZB1011 strain bearing a non-cleavable *lexA* allele (light grey); both strains are harboring reporter plasmids where the promoter of colicins E2, E7, E8, A and K drives the transcription of GFP (labeled ColE2R, ColE7R, ColE8R, ColAR and ColKR, respectively). Controls (reporter plasmid for the chromosomal gene *umuD* (UmuDR) in both strains, and plain cells of both strains) are shown in white. Frequencies of highly fluorescent cells were quantified by flow cytometry and error bars represent the standard deviation of four biological replicates. The activity of all reporter plasmids, except ColE7, was significantly different from exponential to stationary phase cultures (Welch Two-Sided Two Sample t-tests; p-value < 0.05). In the *lexA* background, in exponential phase there was no significant difference in the average frequency of highly fluorescent cells between the UmuD reporter and any of colicin reporters (ANOVA and Tukey's HSD tests; p-value > 0.05); in stationary phase, only the ColK reporter differed significantly from the UmuD reporter (p-value < 0.05), which is

expected since the expression of colicin K is known to be partially independent of LexA and driven by stringent response [1]. Error bars represent the standard deviation of four biological replicates. B) Most cells undergoing spontaneous colicin production are not undergoing stringent SOS response. The promoter of colicin E2 drives the transcription of GFP (labeled ColE2R (GFP)) and the promoter of *umuD* drives the transcription of RFP (labeled UmuDR (RFP)) on the same reporter plasmid replicating in BZB1011 cells. The top row shows the GFP (left) and RFP (right) channels during confocal imaging of a single-cell colony. The bottom row shows the GFP/brightfield (left) and RFP/brightfield (right) fluorescence microscopy images of single cells. C) The graph shows the percentage of single cells in the population expressing GFP only (green bar) or GFP along with RFP (red bar) when the dual *colE2*/*umuD*-reporter plasmid described in (B) was used, as observed by fluorescence microscopy. The percentages have been calculated on a total of at least 11,000 cells. Error bars represent the standard deviation of three biological replicates.

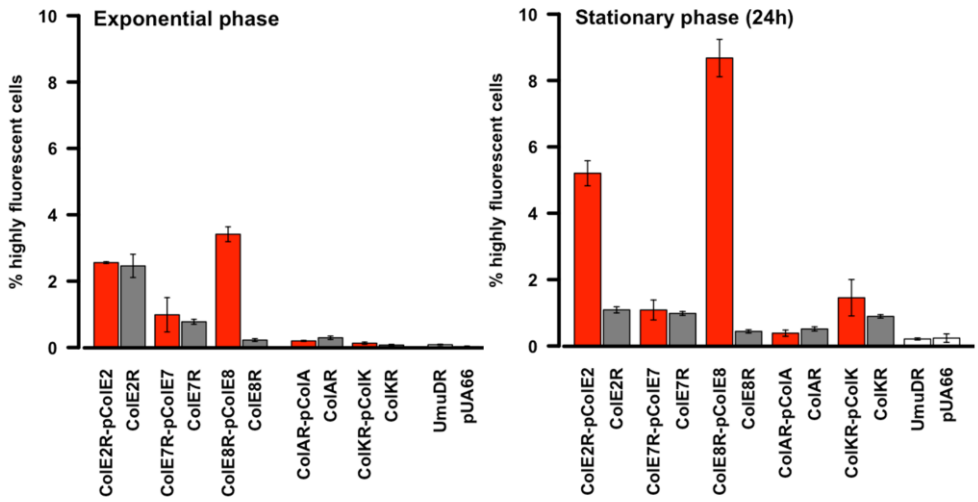

Figure S3 (related to Figure 2). Autoinduction occurs in liquid culture for LexA-regulated colicins. The frequency of highly fluorescent cells was quantified by flow cytometry in liquid cultures in exponential phase (OD600 = 0.3) and stationary phase (24-hour culture) for BZB1011 strains harboring both colicin and reporter plasmids (red), reporter plasmids only (dark grey) and controls (*umuD* reporter or pUA66 vector only) (white). Reporter plasmids for the promoters of colicins E2, E7, E8, A and K or for the chromosomal gene *umuD* are labeled ColE2R, ColE7R, ColE8R, ColAR, ColKR and UmuDR, respectively while the natural plasmids of the same set of colicins are labeled pColE2, pColE7, pColE8, pColA and pColK. Frequencies of highly fluorescent cells are significantly larger (p-value < 0.05) in the presence of colicin plasmids for E8 and K in exponential phase and for E2 and E8 in stationary phase (Welch One-Sided Two Sample t-tests). Error bars represent the standard deviation of four biological replicates.

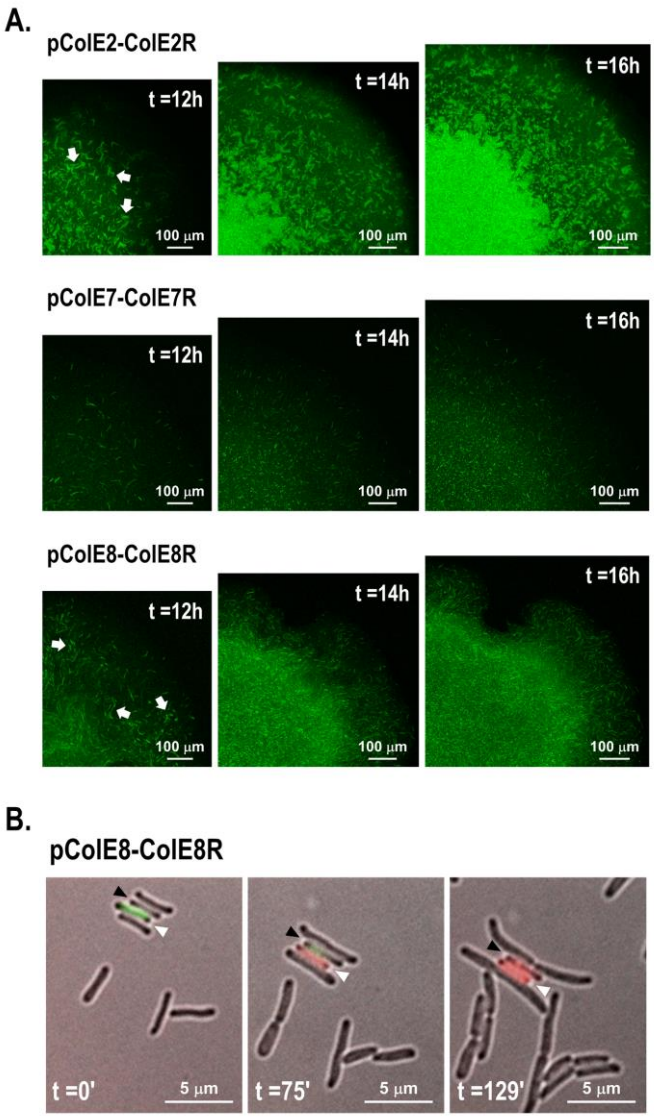

Figure S4 (related to Figure 2). Spatial proximity is crucial for the occurrence of autoinduction. A) The area of colicin producing patches and the frequency of induced cells increase in autoinducing populations (E2 and E8 colicin producers) as colonies become older. For each colicin producer the same single cell-colony was imaged by confocal microscopy after 12, 14 and 16 hours of growth. The promoters of colicins E2, E7 and E8 drive the transcription of GFP on a reporter plasmid (labeled ColE2R, ColE7R and ColE8R, respectively) harbored by BZB1011 cells. Natural plasmids for the same colicins are labeled pColE2, pColE7 and pColE8. White arrows on the left panels indicate patches of autoinducing cells. Over time, the center of the colony becomes saturated with toxins and autoinduction becomes general. B) A time course (Video S1) of BZB1011 cells harboring the colicin E8 plasmid (pColE8) and the corresponding reporter (ColE8R) was recorded by fluorescence microscopy and representative snapshots are shown here. Propidium iodide was added to the medium to monitor cell lysis (cells that become permeable fluoresce red). White arrowheads indicate the bacterial cell which is initially producing colicin (green, first panel) and black arrowheads indicate its neighbor (green, second panel) which is affected by the lysis of this producer (red, second panel). By observing the timescales of the two events in the video and taking into account that significant amounts of colicin leak out prior to the producer

lysing [2], it is likely that the second cell started expressing colicin (green, second panel) as a direct result of exposure to the large amount of DNase toxin produced by its neighbor. Consistent with this, the two cells which are flanking the producing pair elongate and divide irregularly, affected by the presence of the DNase in the medium, however they do not produce colicin. The remaining cells in the field of view are not affected.

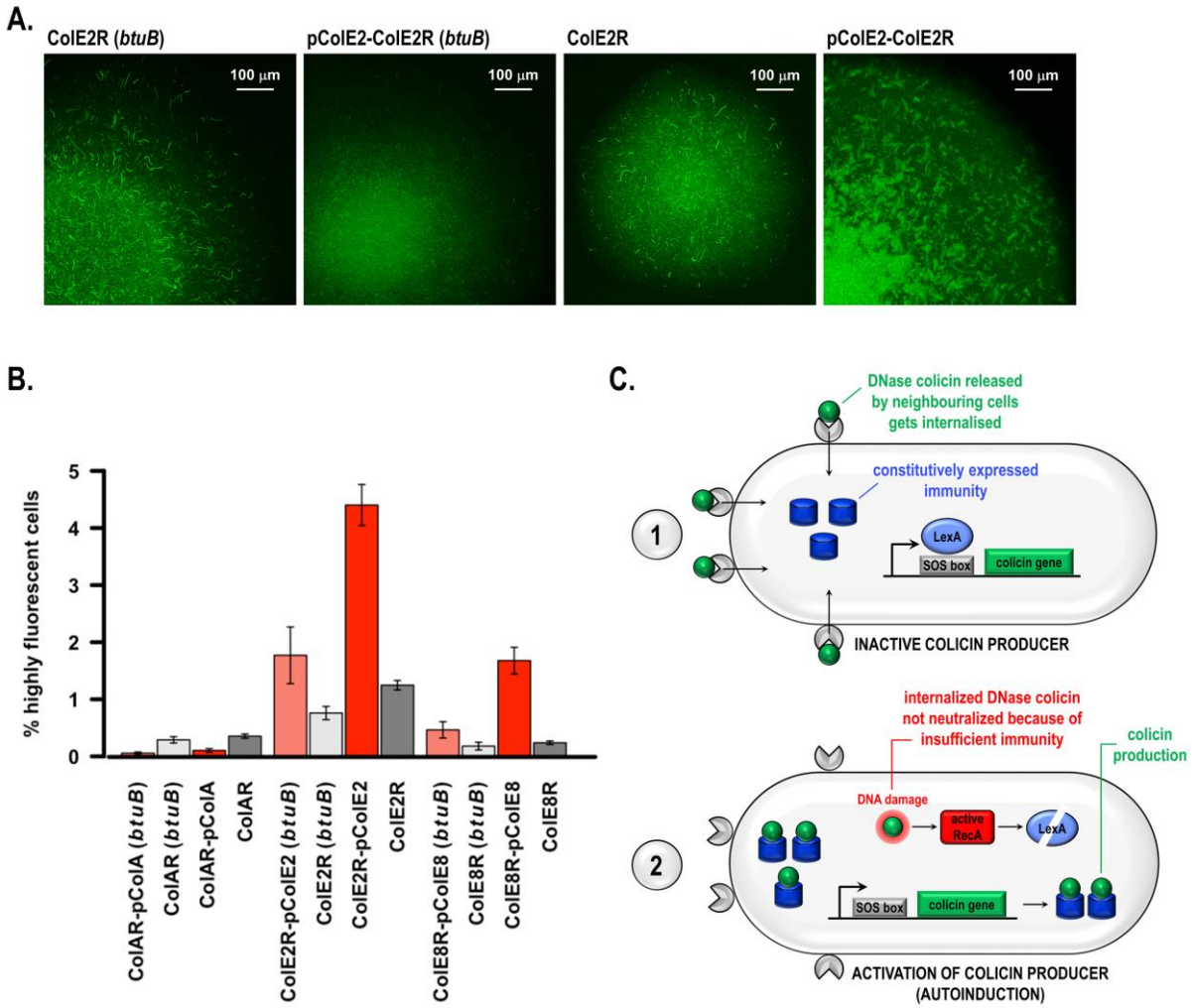

Figure S5 (related to Figure 2). Autoinduction in colicinogenic populations occurs via uptake of external DNase-colicin molecules through their natural colicin receptor. A) Single-cell colonies were imaged by confocal microscopy. A BZB1011 strain where the *btuB* receptor gene is non-functional (due to a transposon insertion) was compared to the wild-type strain. A representative example is shown here; the promoter of colicin E2 drives the transcription of GFP on a reporter plasmid (ColE2R) in cells that do or do not carry the natural colicin E2 plasmid (pColE2). B) Frequencies of cells expressing colicin (cells with high GFP expression) in single-cell colonies of BZB1011 harboring both colicin and reporter plasmids are shown in red for the wild-type strain and in light red for the receptor mutant. Cells harboring colicin reporters only are shown in dark grey for the wild-type strain and in light grey for the receptor mutant. Reporter plasmids for the promoters of colicins A, E2 and E8 are labeled ColAR, ColE2R and ColE8R, respectively, while natural colicin plasmid of colicins A, E2 and E8 are labeled pColA, pColE2 and pColE8, respectively. Highly fluorescent cells were quantified by flow cytometry and error bars represent the standard deviation of four biological replicates. C) Schematic representation of the mechanism of autoinduction. External molecules of a DNase colicin (green spheres) that have been spontaneously released by neighboring clonemates, are uptaken via their natural colicin receptor. If the immunity molecules (blue cylinders) of the recipient producer cell in the same population are not sufficient, the internalized DNase molecules cause DNA damage (see also

Figure 3C and D), generation of single-stranded DNA intermediates as repair is taking place, RecA activation and LexA cleavage. LexA cleavage, in turn, leads to transcription of the colicin gene and to abundant colicin production in the recipient cell.

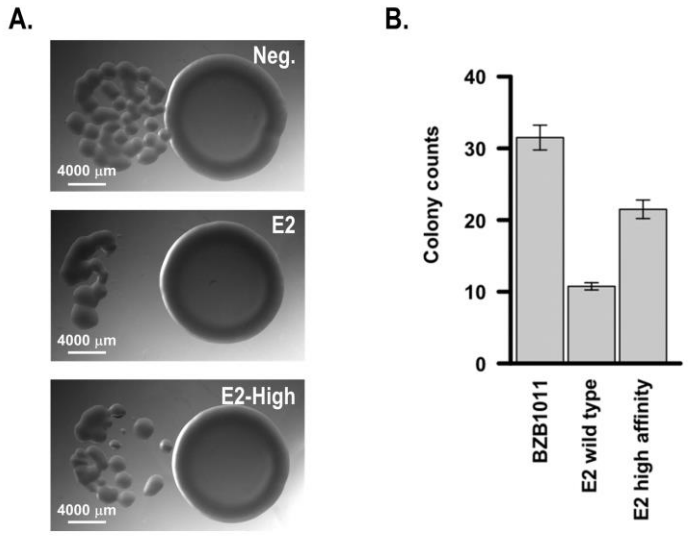

Figure S6 (related to Figure 3). The toxicity conferred by the colicin E2 natural plasmid decreases when the promoter of the toxin contains a high-affinity LexA binding site. A) On the right of each panel normalized dense bacterial populations were spotted. These were BZB1011 non-colicinogenic cells (labeled Neg.), BZB1011 cells harboring a chloramphenicol-resistant wild-type colicin E2 plasmid (labeled E2) or a chloramphenicol-resistant plasmid where the transcription of the *colE2* gene is driven by the promoter with a high-affinity LexA site described in Figure 3A (labeled E2-High). Next to each dense population a 10^6-fold dilution of an overnight culture of a non-colicinogenic population was spotted. The competitions were photographed and surviving colonies were counted after overnight incubation (12 hours) at 37°C. B). The graph shows the average colony counts for each competition experiment. As expected, the toxicity conferred by the plasmid variant containing the high-affinity promoter is significantly lower than that of the wild-type plasmid (p-value<0.01, Student's t-test). Error bars represent the standard deviation of four biological replicates.

SUPPLEMENTAL TABLES

Table S1 (related to Figure 1). Origin and molecular characteristics of colicins which were extensively used in this study.

Colicin plasmid	Original species	Cytotoxicity	Group	Receptor	Import
pColE2	*Escherichia coli*	DNase	A	BtuB	OmpF/TolABQR
pColE7	*Escherichia coli*	DNase	A	BtuB	OmpF/TolABQR
pColE8	*Escherichia coli*	DNase	A	BtuB	OmpF/TolABQR
pColA	*Citrobacter freundii*	Pore	A	BtuB	OmpF/TolABQR
pColK	*Escherichia coli*	Pore	A	Tsx	OmpF/OmpA/TolABQR

Table S2 (related to Methods). Plasmids used in this study.

Name	Description	Source
pColA	Colicin A natural plasmid	[3]*
pColB	Colicin B natural plasmid	[3]*
pColD	Colicin D natural plasmid	[3]*
pColE1	Colicin E1 natural plasmid	[3]*
pColE2	Colicin E2 natural plasmid	[3]*
pColE3	Colicin E3 natural plasmid	[3]*
pColE4	Colicin E4 natural plasmid	[3]*
pColE5	Colicin E5 natural plasmid	[3]*
pColE6	Colicin E6 natural plasmid	[3]*
pColE7	Colicin E7 natural plasmid	[3]*
pColE8	Colicin E8 natural plasmid	[3]*
pColK	Colicin K natural plasmid	[3]*
pColN	Colicin N natural plasmid	[3]*
pUA66	pUA66 vector, promotorless GFP, Kan^R	This study
pUA66-P*colA*::*gfp*	GFP transcribed from *colA* promoter, pUA66	[4]
pUA66-P*colE2*::*gfp*	GFP transcribed from the *colE2* promoter, pUA66	This study
pUA66-P*colE7*::*gfp*	GFP transcribed from the *colE7* promoter, pUA66	[4]
pUA66-P*colE8*::*gfp*	GFP transcribed from the *colE8* promoter, pUA66	This study
pUA66-P*colK*::*gfp*	GFP transcribed from the *colK* promoter, pUA66	[4]
pUA66-P*umuD*::*gfp*	GFP transcribed from the *umuD* promoter, pUA66	This study
pUA66-P*colE2*::*gfp*-P*umuD*::*mrfp1*	GFP transcribed from the *colE2* promoter and mRFP1 transcribed from the *umuD* promoter, pUA66	This study
pGRG25	Plasmid encoding a Tn*7* transposon and arabinose-inducible *tnsABCD*; thermosensitive origin of replication pSC101.	[5]
pUltraGFP-GM	Plasmid providing constitutive mRFP1 expression from a strong synthetic Biofab promoter, p15A origin of replication, $Gent^R$	[6]
pGRG25-P*max*::*gfp*	P*max::gfp* construct from pUltraGFP-GM cloned within the Tn*7* of pGRG25	This study
pGRG25-P*max*::*immE2*	P*max::immE2* construct cloned within the Tn*7* of pGRG25	This study
pColE2-Cm	pColE2 with chloramphenicol resistance inserted downstream of the E2 colicin operon	This study
pColE2-Cm-H	Modified version of pColE2-Cm in which the promoter of the *colE2* gene contains a high-affinity LexA box	This study
pUA66-P*colE2*-L	GFP transcribed from a modified *colE2* promoter containing low-affinity LexA-boxes, pUA66	This study
pUA66-P*colE2*-H	GFP transcribed from a modified *colE2* promoter containing a high-affinity LexA box, pUA66	This study
pSUP404.2	Origin of chloramphenicol resistance cassette	[7]

*Information on the origin of natural colicin plasmids used in this study can be found in [3].

Table S3 (related to Methods). Bacterial strains used in this study.

Name	Description	Source
JJC443	*lexAind3 malF*::Tn*10*	[8]
BZB1011	W3110, *gyrA*, Str^R	[9]
BZB1011 (*lexA*)	BZB1011, *lexAind3*	This study
BZB1011 (*btuB*)	BZB1011, *btuB*	This study
BZB1011 (*gfp*)	BZB1011 Tn*7*::P*max::gfp*	This study
BZB1011 (*immE2*)	BZB1011 Tn*7*::P*max*::*immE2*	This study

Table S4 (related to Methods). Oligonucleotide primers for cloning used in this study.

Name	DNA sequence (5´-3´)
P1	AACTCGAGAGACCTGGCATGAGTGGAAG
P2	AAGGATCCCTACACCAAGCCCGGTCG
P3	AACTCGAGCCGTCAACTCGGATTTAATCAG
P4	AAGGATCCGAACCATCAGAAGCACCACC
P5	AACTCGAGTGTAATGAAACTCCTGTTTTACAAC
P6	AAGGATCCGGATCAACAGTTGATTCAGATC
P7	ACTACTTGCCATATGTATATCTCCTTCTTAAATCTAGAGG
P8	GGAGATATACATATGGCAAGTAGTGAAGACGTTATC
P9	GGCTCGAGGGAAGCCTGCATAACGCG
P10	AGGCGATCGGAGCTCCTTTCGCTAAGGATGATTTCTGG
P11	AGGGCGGCCGCTTAATTAAGAGCTCGAGTCAGTGAGCGAGGAAGC
P12	GGTCAATAGCGGCCGCCACTCTAGCACCTAGGAGGCGCGCCGGATCCAAC AGGGTTCTCGAG
P13	ACCACGCGTATTACCGCCTTTGAGTGAGCTG
P14	CGATCGCGTATCACGAGGCAGAATTTCAG
P15	CGAGTGGCGGCCGCTATTGACCCGGGCCCATCTGATTAATTAACTTATTTT GCATCCATGAATCTATTATAGG
P16	AGGGCGGCCGCAAATCAACACCGCAACACACGAATTATCATTTATGGAAC TGAAACATAGTATTAGTGATTATACCGAG
P17	AAAGGCGCGCCTCAGCCCTGTTTAAATCCTGACTTACC
P18	AGCGGCCGCATCACCTAAATATGACCGTTACTTTCTTC
P19	AGCGGCCGCGTTGCATAACTATGCACGAAAGC
P20	GAAAAAACGATGACGGGTACTTTTTGTACTGTATATAAAACCAGTGGTTT TATGTACAGTATTAATCATGTAATTAATTGTTTTAACGC
P21	GCGTTAAAACAATTAATTACATGATTAATACTGTACATAAAACCACTGGT TTTATATACAGTACAAAAAGTACCCGTCATCGTTTTTTC
P22	GAAAAAACGATGACGGGTACTTTTTGGTCTGTACATAAAACCAGTGATTT TATGTACAGTATTAATCATGTAATTAATTGTTTTAACGC
P23	GCGTTAAAACAATTAATTACATGATTAATACTGTACATAAAATCACTGGT TTTATGTACAGACCAAAAAGTACCCGTCATCGTTTTTTC
P24	CAAAAAGTACCCGTCATCGTTTTTTCTG
P25	TTAATCATGTAATTAATTGTTTTAACGCTTAAAAGAGG

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