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# Structured habitats and the evolution of anticompetitor toxins in bacteria

(allelopathy/interference competition/altruism/frequency-dependent selection/colicin)

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**ABSTRACT** **We demonstrate that in liquid cultures, defined in this study as a mass habitat, the outcome of competition between *Escherichia coli* that produce an antibacterial toxin (colicin) and sensitive *E. coli* is frequency dependent; the colicinogenic bacteria are at an advantage only when fairly common (frequencies in excess of $2 \times 10^{-2}$). However, we also show that in a soft agar matrix, a structured habitat, the colicinogenic bacteria have an advantage even when initially rare (frequencies as low as $10^{-6}$). These contrasting outcomes are attributed to the colicinogenic bacteria's lower intrinsic growth rate relative to the sensitive bacteria and the different manner in which bacteria and resources are partitioned in the two types of habitats. Bacteria in a liquid culture exist as randomly distributed individuals and the killing of sensitive bacteria by the colicin augments the amount of resource available to the colicinogenic bacteria to an extent identical to that experienced by the surviving sensitive bacteria. On the other hand, the bacteria in a soft agar matrix exist as single-clone colonies. As the colicinogenic colonies release colicin, they kill neighboring sensitive bacteria and form an inhibition zone around themselves. By this action, they increase the concentration of resources around themselves and overcome their growth rate disadvantage. We suggest that structured habitats are more favorable for the evolution of colicinogenic bacteria.**

A number of phylogenetically distinct groups of bacteria produce toxic substances, known collectively as bacteriocins, that can kill members of the same or closely related species (1). The colicins, the most extensively studied class of bacteriocins, are produced by the bacterium *Escherichia coli* and other members of the family Enterobacteriaceae (for reviews see refs. 1–4). In general: (*i*) at any given time only a small fraction of the colicinogenic population actually produces colicin; (*ii*) the production of the colicin results in the death of the producing (induced) cells—i.e., lethal synthesis; (*iii*) colicinogenic bacteria not producing colicin are immune to the colicin released by the induced cells; and (*iv*) the characters of colicin production and immunity are determined by plasmid-borne genes. Colicin can kill sensitive bacteria by many varied mechanisms. For example, colicin K acts by deenergizing the cell membrane, colicin E2 inhibits DNA synthesis, whereas colicin E3 inhibits protein synthesis. In all cases, however, the killing action requires the initial adsorption of a colicin particle onto specific receptors present on the surface of sensitive bacteria. Multiple adsorptions per individual bacterium are possible, but a single hit is sufficient to kill. Sensitive bacteria, in turn, can mutate to resistance through the alteration or loss of the receptors. Resistance differs from the immunity of colicinogenic bacteria in that the latter results from the synthesis of an intracellular inhibitor that neutralizes a colicin particle after it has adsorbed to a colicinogenic bacterium. It is generally believed that bacteria have evolved the production of such toxins as an anticompetitor mechanism (2, 4, 5).

The process of colicin production by lethal synthesis is of evolutionary interest since it requires that selection operates on a trait that is lethal to the individual manifesting the trait. From this point of view, colicin production has been interpreted to be an altruistic character (6), and the conditions necessary for its evolution are analogous to those required for the evolution of interference competition in general. Colicin production should not be favored in situations of mass competition such as those described by the classical models of Volterra and Gause (7), but it should be favored in instances in which individual or group identity is preserved such that the interfering genotypes gain a larger share of the resulting benefits (8).

In an effort to determine the conditions under which colicinogenic bacteria would be favored by selection, we have used laboratory populations of *E. coli* to study competition between populations of a sensitive strain and a colicinogenic, otherwise isogenic, strain carrying a ColE3 plasmid. Although some colicinogenic plasmids are capable of mediating their own transfer through conjugation, the ColE3 plasmid is nonconjugative (9). In this study, because we have excluded the presence of conjugative plasmids and transducing viruses, the ColE3 plasmid cannot be transferred to the sensitive population and it can be treated as a permanent character of the colicinogenic population. We have investigated the behavior of our competing populations in what we have defined as mass and structured habitats. A mass habitat is one in which individuals affect equally the environment of every other individual and is represented in this study by well-mixed serial transfer liquid cultures. A structured habitat is defined as one in which individuals affect differently the environment of different individuals, as on a surface where closer neighbors might interact more intensely. For the latter, we cultured our bacterial populations on serial transfer agar plates where the bacteria were allowed to grow into distinct colonies, hence preserving clonal and individual identity. Our results suggest that structured habitats are much more favorable for the evolution of colicinogenic bacteria.

## MATERIALS AND METHODS

Total numbers of bacteria were estimated from colony counts made on broth agar plates. In the competition experiments between sensitive and colicinogenic bacteria, the numbers of the latter were determined by sampling on broth agar plates previously spread with 0.1 ml of crude colicin E3 to prevent the growth of the sensitive bacteria. To insure that the colicinogenic bacteria sampled on those plates were not simply resistant, the bacteria were routinely assayed for colicin production. In the

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competition experiments between resistant and colicinogenic bacteria, a multiple overlay plate was used to distinguish between the two competitors. These plates were prepared by following the procedure described by Fredericq (2) except that two 2-ml layers of soft agar were used instead of a single 10-ml second layer. With this multiple overlay technique, it is possible to detect a single colicinogenic bacterium in a sample of $10^6$ bacteria. All of the techniques used for assaying colicinogenic and resistant bacteria are detailed by Fredericq (2).

The sensitive strain used for this study was a plasmid-free *E. coli* B strain originally obtained from S. Lederberg. The colicinogenic strain was obtained by transferring the nonconjugative ColE3 plasmid (provided by D. Helinski) into this plasmid-free *E. coli* B strain by mobilization with a ColIb conjugative plasmid (provided by J. Campbell), selecting for colicin E3 immunity, and then screening for colicin E3 production. The colicin E3-resistant strain harboring the ColE3 plasmid was constructed by transferring the plasmid into a colicin E3-resistant strain by mobilization with the ColIb plasmid and then screening, without selection, for the desired transconjugant by a multilayer overlay. None of the resulting ColE3 strains manifested the colicin Ib phenotype or revealed the presence of the ColIB plasmid when lysates were examined on agarose gels for low molecular weight DNA (10). The resistant strain was isolated as a mutant by plating about $10^8$ sensitive bacteria on a broth agar plate spread with colicin E3.

All of our experimental populations were grown in a minimal medium identical to that described by Levin *et al.* (11), except that NaCl was added at 250 $\mu$g/ml. Glucose, the limiting resource, was added at the concentrations indicated below.

Our liquid medium serial transfer cultures were modified versions of those used by Atwood *et al.* (12) and Levin (13). The bacterial populations were maintained in 50-ml Erlenmyer flasks containing 5 ml of minimal media with glucose at 300 $\mu$g/ml. These cultures were initiated by introducing colicinogenic and sensitive bacteria grown in overnight monocultures with the same minimal medium. The bacteria were introduced at various starting frequencies but always at a total density of $4 \times 10^5$ cells per ml. After these cultures had been incubated for 24 hr at 37°C with agitation, samples were taken from the resulting stationary-phase cultures and plated to estimate the total density of bacteria and the relative frequencies of colicinogenic and sensitive bacteria. Under these culture conditions, the resulting stationary-phase cultures have a density of approximately $4 \times 10^8$ cells per ml. New transfer cycles were initiated by adding 0.005 ml of stationary-phase cultures to flasks containing 5 ml of fresh medium. This process of sampling and initiating new cultures was continued for a number of transfers.

Our structured habitat populations were grown in a soft agar matrix spread over an agar plate and were maintained by serial transfer in a manner similar to that used for the liquid cultures. To initiate these soft agar cultures, both types of bacteria (again from overnight liquid monocultures) were first introduced into 3 ml of molten (50°C) soft agar (minimal medium with 0.7% agar). The soft agar suspension was then mixed and poured onto Petri plates (100 × 15 mm) containing about 30 ml of hardened bottom agar (minimal medium with 1.3% agar). The 3 ml of soft agar contained a total of 10 mg of glucose, but no glucose was present in the bottom agar. The relative frequencies of colicinogenic bacteria in each initial inoculum varied but in all cases the total number of bacteria was about $10^7$ cells. After an incubation period of 24 hr at 37°C, the soft agar matrix was scrapped into bottles containing 20 ml of the minimal medium (without glucose or agar) and shaken vigorously for approximately 5 min to free the bacteria from the soft agar. The relative numbers of colicinogenic and sensitive bacteria were estimated from these bacterial suspensions and successive serial transfer cycles were initiated by adding 0.2 ml of a $10^{-1}$ dilution of the suspensions to 3 ml of fresh soft agar (with 10 mg of glucose) and pouring that mixture onto fresh bottom agar plates. The 10 mg of glucose yielded after incubation a stationary-phase population of about $10^{10}$ bacteria per plate. Because each soft agar plate was initially seeded with a total of $10^7$ bacteria, each bacterium grew on the average into a colony consisting of about $10^3$ individuals.

## RESULTS

In Fig. 1 we present the results of a series of liquid competition experiments. The outcome is clearly frequency dependent. When the relative frequency of colicinogenic bacteria was above an unstable equilibrium point (about $2 \times 10^{-2}$) their frequency rapidly increased and the population of sensitive bacteria appeared to be eliminated. When the frequency of colicinogenic bacteria was below the unstable equilibrium point, they were displaced by the sensitive bacteria, suggesting that, at those low frequencies, the killing effect of the colicin must have been minimal and that the colicinogenic bacteria had an intrinsic growth rate disadvantage, presumably due to the costs of lethal synthesis, immunity, and carrying the colicinogenic plasmid. In several cases around the fourth or fifth transfers, the rate of decline of the colicinogenic bacteria increased precipitously. We attribute this sudden change to the effects of "periodic selection" (12) bringing to fixation in the sensitive population a new clone that is better adapted to the culture conditions than both the original sensitive and colicinogenic populations. The new clone has a greater chance of appearing in the sensitive population because, at frequencies below the unstable equilibrium point, those populations were initially larger. We tested this periodic selection hypothesis by isolating "transferred" colicinogenic and sensitive bacteria at the end of the seventh transfer from the culture initiated with a starting frequency of $9 \times 10^{-3}$ in Fig. 1 and then culturing them in competition with the original colicinogenic and sensitive stocks. The results of these competition experiments (not presented) clearly supported our hypothesis; the transferred and the original colicinogenic clones

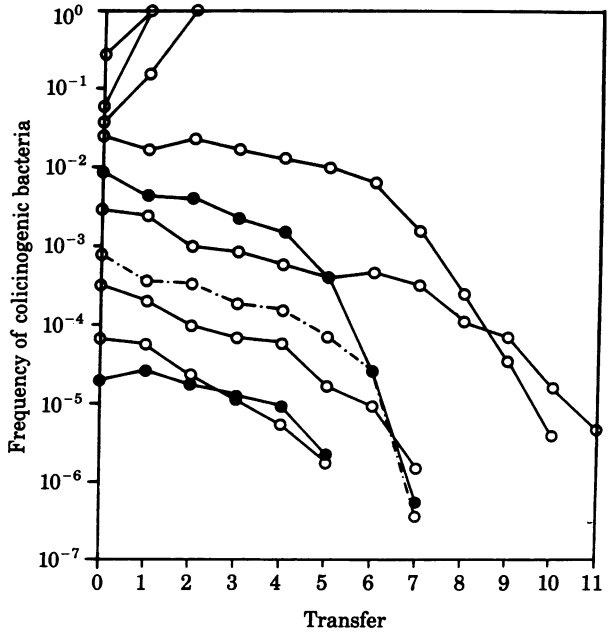


FIG. 1. Changes in frequency of colicinogenic bacteria in competition with sensitive bacteria in liquid medium cultures.

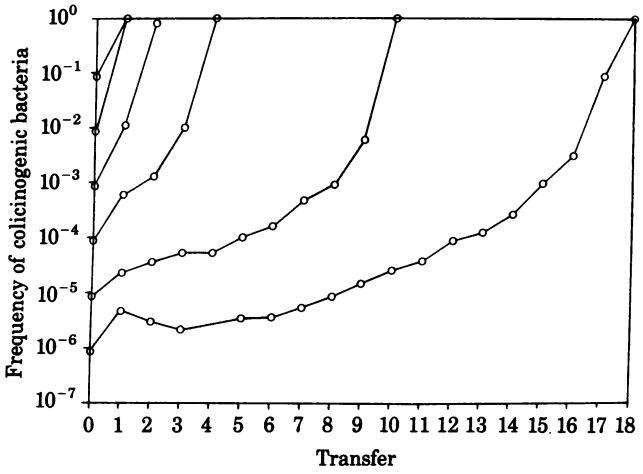


FIG. 2. Changes in frequency of colicinogenic bacteria in competition with sensitive bacteria in soft agar plates.

were equally fit, but the transferred sensitive clone was competitively superior to the original sensitive clone.

In Fig. 2 we present the results of a series of soft agar competition experiments. Selection for colicinogenic bacteria was still frequency dependent—the relative rate of increase of the colicinogenic bacteria increased with their relative frequency—but the colicinogenic bacteria could now increase from frequencies four orders of magnitude lower than the value of the unstable equilibrium point observed in the liquid culture system.

The colicinogenic and sensitive bacteria formed distinct colonies on our soft agar plates, and there were clear differences between them. As it can be seen in Fig. 3, the colonies of colicinogenic bacteria were surrounded by an inhibition zone free of sensitive bacteria and they (mean diameter ± SEM = 25.3 ± 1.43 $\mu$m, $n$ = 17) were significantly larger than the colonies of sensitive bacteria (mean diameter ± SEM = 4.8 ± 0.43 $\mu$m, $n$ = 64). In addition, the coefficient of variation (SD × 100/mean) for the colicinogenic colonies (23.3%) was markedly smaller than that for the sensitive colonies (70.8%).

Although colicin E3-resistant mutants can be readily isolated from our populations of sensitive bacteria, resistant bacteria never increased in frequency in any of our liquid or soft agar cultures. One possible explanation for this result is that the resistant bacteria were competitively inferior to the colicinogenic bacteria. We tested this hypothesis by allowing the colicinogenic strain to compete with a resistant strain in soft agar plates, and the results are shown in Fig. 4*A*. As it is evident, the colicinogenic bacteria were clearly at an advantage, rapidly displacing the resistant strain.

To control for the possibility that the advantage of the colicinogenic bacteria relative to the sensitive bacteria on the soft agar plates could have resulted from a higher intrinsic growth rate (although we had already found the converse to be true in liquid cultures) we cultured on soft agar plates in pairwise competition a colicin E3-resistant strain of *E. coli* B and a second isogenic resistant strain that also carried the ColE3 plasmid. We chose to use a resistant strain to eliminate the inhibitory effects of colicin and made the colicinogenic strain resistant, in addition to being immune, to control for the fitness difference between immunity and resistance (see above). The results of these competition experiments, presented in Fig. 4*B*, contradict the possibility of an intrinsic growth rate advantage for the colicinogenic bacteria relative to the sensitive bacteria. In two replicates, the colicinogenic bacteria were unable to increase in numbers from starting frequencies of $10^{-4}$ and $10^{-5}$. In fact, the colicinogenic–resistant strain was at a disadvantage relative to the resistant strain, indicating that the former must incur a cost for lethal synthesis, immunity, and bearing the plasmid in the soft agar plates, as in liquid cultures.

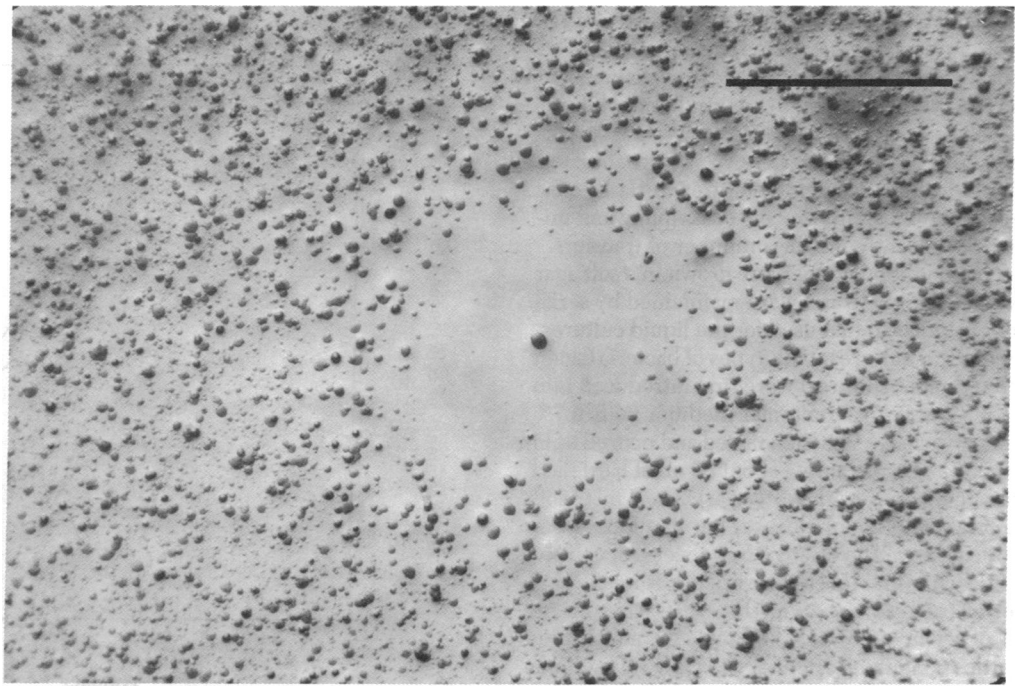

FIG. 3. Photomicrograph showing a magnified section of a soft agar plate with colonies of sensitive bacteria and one colony of colicinogenic bacteria surrounded by an inhibition zone. Scale bar equals 500 $\mu$m.

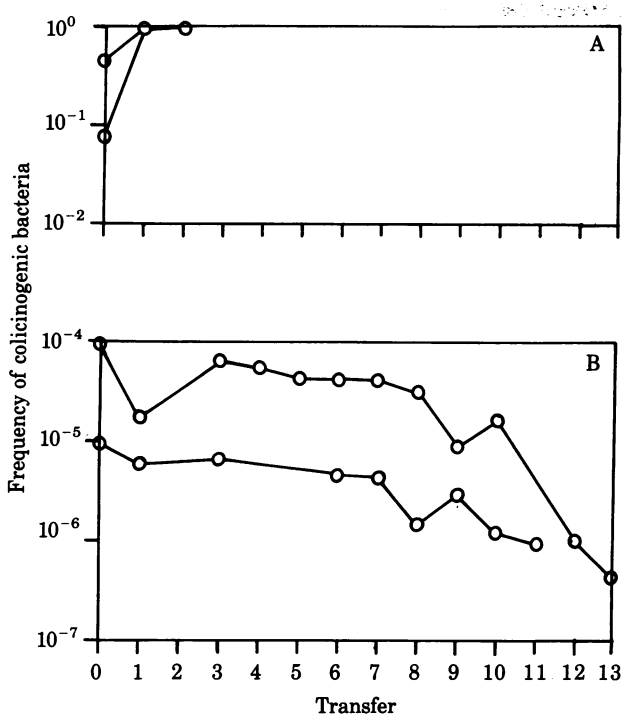


FIG. 4. (*A*) Changes in frequency of colicinogenic bacteria in competition with resistant bacteria in soft agar plates. (*B*) Changes in frequency of colicinogenic–resistant bacteria in competition with resistant bacteria in soft agar plates.

## DISCUSSION

Our results show that in liquid cultures, defined in this study as a mass habitat, colicinogenic bacteria have an overall selective advantage relative to sensitive bacteria only when the former are common. This frequency-dependent outcome is consistent with some earlier liquid culture competition results with other strains of colicinogenic and sensitive bacteria and is attributed to the interplay between two opposing factors (14, 15). As indicated by Adams *et al.* (15), colicinogenic bacteria have a lower intrinsic growth rate than sensitive bacteria and this disadvantage, presumed to result from the loss of individuals through lethal synthesis of colicin and from the energetic costs of maintaining immunity and carrying the colicinogenic plasmid, accounts for the decrease of the colicinogenic bacteria when they are rare. Colicinogenic bacteria overcome this disadvantage when common because at those higher frequencies they are more numerous and, as a result, produce a sufficient amount of colicin to adequately inhibit the growth of the sensitive population.

When we cultured sensitive and colicinogenic bacteria on plates in a soft agar matrix, a structured habitat, we observed an outcome markedly different from that obtained in liquid cultures; the colicinogenic bacteria had an overall competitive advantage even when rare. Both types of bacteria formed distinct colonies on these plates, but the colicinogenic colonies, surrounded by an inhibition zone, grew to a larger size. We considered two alternative hypotheses to account for the success of the colicinogenic bacteria in our soft agar plates: (*i*) in soft agar the colicinogenic bacteria could have a much higher intrinsic growth rate than the sensitive bacteria, in spite of the fact that we had already observed a slightly lower growth rate for the former in liquid cultures; (*ii*) the overall advantage of the colicinogenic bacteria in soft agar resulted from the manner in which the bacteria and the resources were partitioned in that habitat, still assuming that the colicinogenic bacteria had a slightly lower intrinsic growth rate. That is, in the liquid cultures, because all of the bacteria would be randomly distributed with respect to their genotypes, when the colicinogenic bacteria were rare, the killing of sensitive bacteria by the colicin augmented the amount of glucose (the limiting resource) available to the surviving sensitive bacteria to an extent identical to that experienced by the colicinogenic bacteria. On the other hand, the interaction between the two populations of bacteria on soft agar plates can be considered at a group level in the form of intercolony competition. The colinicogenic colonies, by releasing colicin, generate inhibition zones around themselves. Although individual colicinogenic bacteria within any single colicinogenic colony die through lethal synthesis of colicin, in a structured habitat, their action benefits the surviving members of their own colony more than it benefits the surviving sensitive bacteria outside the inhibition zones. Those surviving colicinogenic bacteria, by virtue of their proximity, have primary access to the pool of glucose inside the inhibition zones. A structured habitat could favor the colicinogenic bacteria by allowing them to acquire a larger share of the benefits resulting from the action of the colicin and thus overcome their intrinsic growth rate disadvantage.

We tested these two hypotheses by pairing in competition on soft agar plates a colicinogenic–resistant strain and an otherwise isogenic resistant strain. The resistant phenotype was used to eliminate the inhibitory effects of colicin and the colicinogenic strain was made resistant, in addition to being immune, to control for the fitness difference between immunity and resistance (see below). Without the benefit of the action of colicin, by the first hypothesis, the colicinogenic–resistant bacteria should still have been favored, whereas, by the second hypothesis, the opposite would have been expected. As it turned out, the colicinogenic–resistant bacteria were at a disadvantage relative to the resistant bacteria, clearly giving grounds for rejecting the first hypothesis.

In addition, our second hypothesis for the overall success of the colicinogenic bacteria on soft agar plates is supported by several other observations.

First, because the resource sequestering mechanisms proposed by this second hypothesis should provide to every individual colicinogenic colony a net advantage over the entire sensitive population, one would not anticipate an unstable equilibrium point for the outcome of competition between these two bacteria in a structured habitat. In this study, the colicinogenic bacteria increased in frequency on the soft agar plates even when there were only 15 colicinogenic colonies per plate (a relative frequency of $10^{-6}$), and it is likely that they could still increase if their density was reduced to one colony per plate.

Second, although the colicinogenic bacteria had an advantage at all frequencies in the soft agar experiments, their rate of ascent was still frequency dependent; it increased with their frequency. Our second hypothesis would attribute this frequency-dependent effect to a less than perfect containment of glucose by the inhibition zones. By generating an inhibition zone, a colicinogenic colony gains access to an extra supply of glucose, but some of that glucose inevitably diffuses away before it is consumed. When the frequency of colicinogenic bacteria is low and their colonies are spaced far apart, that glucose escapes to bordering surviving colonies of sensitive bacteria and is lost by the colicinogenic population. As the frequency of colicinogenic bacteria is increased, a synergistic effect is achieved because their inhibition zones begin to overlap and the escaping glucose is now recovered as it diffuses into another inhibition zone.

Beyond a point, this synergistic effect should decrease since the total amount of resources covered by these zones is inversely related to their degree of overlap. Such a decrease was not observed in our present study, possibly because when inhibition zones overlapped to that extent, most or all of the plate was covered by the inhibiton zones and most or all of the sensitive population was killed.

Third, this resource sequestering hypothesis can also account for the fact that in soft agar plates the variation in the colony size of colicinogenic bacteria (coefficient of variation = 23.3%) was less than that of sensitive bacteria (coefficient of variation = 70.8%). The size of an individual colony on a soft agar plate is primarily dependent on the amount of glucose available to it. For the colicinogenic colonies, the supply of this resource should roughly determined by the area of their inhibition zones. For the surviving sensitive colonies, this supply should be proportional to a considerably more variable factor, the proximity of competing colonies.

Adams *et al.* (15) in a study with another colicin, E1, reported that a resistant noncolicinogenic mutant generated by the sensitive population eventually replaced the colicinogenic population. That study examined populations grown in liquid (chemostat) cultures, and it suggested that the resistant mutants were competitively superior to the colicinogenic bacteria. Although noncolicinogenic mutants resistant to colicin E3 can be readily isolated from our populations of sensitive bacteria, these mutants never increased in frequency in any of our liquid or soft agar cultures. We attribute our results to the fact that colicin E3-resistant bacteria are competitively inferior to colicin E3 colicinogenic bacteria. When we compared those bacteria in pairwise competition on soft agar plates, the resistant strain was rapidly displaced. Although resistance and immunity are similar phenotypically, the two result from distinctively different genetic mechanisms. Resistant bacteria lack the specific receptors necessary for the adsorption, and hence action, of colicin. Colicinogenic bacteria, like sensitive bacteria, still possess those receptors, but they are immune because they produce an intracellular inhibitor that neutralizes a colicin particle after it has adsorbed. It is not unexpected that the loss of a membrane receptor with other possible functions could lead to a decrease in fitness. Bacteriophage resistance in *E. coli* results from the loss of similar membrane receptors, and it can lead to dramatic reduction in competitive fitness (16). In our present study, it appears as if the cost of losing the colicin E3 receptor is more than that of the combined effects of immunity, lethal synthesis, and bearing the ColE3 plasmid.

As indicated earlier, it is generally believed that colicins and other bacteriocins evolved as an anticompetitor mechanism (2, 4, 5). This ecological role of colicins, however, has been questioned on the basis of the results of some *in vivo* studies in which populations of sensitive and colicinogenic bacteria were found to coexist in the same host (17, 18). Established populations of colicinogenic bacteria could not exclude sensitive bacteria, and if sensitive bacteria were allowed to establish first in a host, colicinogenic bacteria could invade but could not displace the sensitive population. The results of our experiments suggest that the ambiguity of these *in vivo* studies could be accounted for if *in vivo* habitats have both mass and structured components. It would be of interest to test whether the colicinogenic strains, if cured of their colicinogenic plasmids, could still invade an established sensitive population.

The facts that colicinogenic bacteria must have been rare at the time of their origin and that they cannot increase from low frequencies in a mass habitat make it likely that structured habitats are necessary for the evolution of colicin. This requirement was originally appreciated by Reeves (1) and could well be relevant to the evolution of anticompetitor (allelopathic) toxins in general. The possibility that naturally occurring populations of bacteria are structured by their habitats is supported by the fact that most species of bacteria have specific adaptations for adhesion to surfaces (19). The attachment of bacteria to surfaces, most likely an adaptive response to a variety of other factors, would facilitate the formation of clonal populations, which in the case of colicinogenic bacteria allows for the mechanism of lethal synthesis of colicin to be of advantage to the genotypes manifesting that character. In fact, the ease with which asexually reproducing microorganisms lend themselves to the formation of monoclonal populations in structured habitats could well account for the occurrence of other forms of individual altruism in microbial systems (20–22).

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