Evolution, 53(4), 1999, pp. 1019–1027

THE PHENOTYPIC AND FITNESS EFFECTS OF COLICIN RESISTANCE IN *ESCHERICHIA COLI* K-12

MICHAEL FELDGARDEN[1] AND MARGARET A. RILEY[2]
[1]*Biology Department, Wesleyan University, Hall-Atwater Laboratories, Room 257, Lawn Avenue, Middletown, Connecticut 06459-0170*
E-mail: mfeldgarden@mail.wesleyan.edu
[2]*Department of Ecology and Evolutionary Biology, Yale University, P.O. Box 208104, New Haven, Connecticut 06521-8104*
E-mail: riley@beagle.biology.yale.edu

Abstract.—Previous studies indicate that most natural isolates of *Escherichia coli* are resistant to most or all colicins (antibiotics produced by *E. coli*) when assessed in the laboratory. Additionally, resistance to different colicin types appears to arise in a nonindependent manner. One possible mechanism to explain this nonindependence is pleiotropy: Multiple resistances are selected after exposure to a single colicin. This study, which was designed to address the role of pleiotropy in the generation of colicin resistance, revealed that 96% of colicin resistant mutants were resistant to two or more colicins. Mutational class was important because putative translocation mutants (Tol pathway mutants) resisted fewer colicins than putative receptor mutants. To determine whether colicin resistance is costly, the effects of colicin resistance mutations on maximal growth rate in a rich medium were also examined. Relative to the sensitive ancestor, translocation mutations lowered maximal growth rates by 17%, whereas putative receptor mutations did not significantly lower growth rates. Thus, when nutrients are abundant, the most advantageous forms of colicin resistance may not impose a cost. The ecological consequences of pleiotropic colicin resistance could involve population cycling between colicin sensitivity and resistance. Additionally, if the cost of resistance depends on the environment, ecological diversification could result.

Key words.—Colicin resistance, colicins, fitness effects, pleiotropy.

Received July 13, 1998. Accepted February 10, 1999.

Pleiotropy is thought to be a critical evolutionary condition affecting the evolution of diverse phenomena such as senescence (Service et al. 1988), niche divergence (Levins 1968; Lynch and Gabriel 1987), host-pathogen interactions (Gillespie 1975), and pesticide resistance (Chevillon et al. 1997). Pleiotropy is often assumed to be antagonistic, in that a novel phenotype (e.g., toxin resistance) is deleterious in the absence of selective agent. In this case, there is a trade-off between the original and the new phenotype that is mediated by the presence or absence of the selective agent. Antagonistic pleiotropy could then lead to polymorphism and ecological divergence if the environment is heterogeneous in space or time (Holt and Gaines 1992; Kawecki 1995). It is less clear, however, what the consequences of pleiotropy will be if multiple advantageous phenotypes result. For example, resistance to a toxin could confer resistance to multiple toxins that have not yet been encountered. Pleiotropic mutations then could decouple the selective forces that result in the origin of a trait from the selective forces that presently maintain a trait (i.e., exaptations sensu Gould and Vrba 1982).

To determine the consequences of pleiotropy when pleiotropic effects are complex, one must assess the potential range of phenotypic effects due to pleiotropy along with the fitness effects of pleiotropy in nonfavorable environments. One system that lends itself to such study is colicin resistance in *Escherichia coli*. Colicins are plasmid-encoded protein antibiotics produced by, and active against, *E. coli* and related species. Colicins provide an ideal model system for attempting to understand the evolution and maintenance of antagonistic competition. Levels and patterns of colicin protein diversity suggest that colicins experience strong selection to diversify into novel types; thus, colicins appear to play a significant role in mediating *E. coli* population dynamics (Riley 1993 a,b; Tan and Riley 1996, 1997).

Experimental studies performed in laboratory microcosms have provided further evidence for the potential importance of colicins in population dynamics. Colicin-producing strains have been shown to invade sensitive, non-colicin-producing populations rapidly, although solid surfaces could be invaded with lower initial frequencies of producers than liquid media (Chao and Levin 1981). Tan and Riley (1997) showed that novel colicin producers can invade an already established colicinogenic population, even with low initial frequencies. Finally, multiple population surveys reveal that colicin production occurs at high frequencies (5–50% of strains; Riley and Gordon 1996). The ubiquity of colicin production has been assumed to indicate the importance of colicinogeny in natural environments.

Unlike colicin production, far less attention has been focused on the population-level response to high levels of colicin production. Colicin resistance is even more ubiquitous than colicin production, with over 90% of the strains surveyed resistant to most colicins, and approximately 25–40% of the strains resistant to all colicins examined (Riley and Gordon 1992, 1996; Feldgarden and Riley 1998).

Colicin resistance has been investigated at the molecular level (Fredericq 1957; Zinder 1973; Davies and Reeves 1975a,b; Maher et al. 1989; Whelan et al. 1995). There are three basic protective mechanisms against colicins. The first is immunity that involves the production of a specific protein that binds to and renders inactive a particular colicin. The second protective mechanism is resistance. Resistant cells have altered colicin receptors and are unable to bind the colicin molecule (Davies and Reeves 1975a,b; Pugsley 1984; James et al. 1996). Tolerance occurs when the translocation pathways that move the colicin into the cell have been altered, such that colicin import is highly inefficient or impossible

(Davies and Reeves 1975a,b; Pugsley 1984; James et al. 1996). Molecular and genetic studies have demonstrated that exposure to one colicin can result in resistance to multiple colicins (Davies and Reeves 1975a,b). A single mutation in either the colicin-binding receptor or translocation pathway can result in resistance to one, some, or all of the colicins that use that receptor or pathway (Pugsley 1984; Webster 1991).

Surveys of colicin resistance among natural isolates suggest that the pleiotropic generation of colicin resistance might explain the observed high levels of naturally occurring resistance (Riley and Gordon 1996; Feldgarden and Riley 1998). First, natural populations harbor significantly higher levels of resistance than would be predicted from the currently segregating colicins. Second, two collections that currently produce different colicins possess phenotypically indistinguishable resistance patterns. In either collection, the high levels of resistance may result from historical exposure to colicins that are no longer segregating. Alternatively, current resistance profiles may reflect recent selection for resistance with multiple resistances arising from single colicin selection events.

It has been assumed that colicin resistance is costly (Levin 1988). Thus, it is predicted that high levels of resistance should be costly to maintain unless there is continuous exposure to colicins. Receptor-based resistance in some cases can lower growth rates when certain nutrients are limiting (Pugsley and Reeves 1976); tolerance can also disrupt the integrity of the cell membrane, making the cell more susceptible to environmental perturbations (Webster 1991). However, no experiment has been specifically designed to assess the fitness effects of colicin resistance.

Here we investigate the patterns of production of colicin resistance by generating and phenotypically characterizing 219 colicin resistant mutants exposed to one of 11 different colicins. In addition, a subset of these mutants is assessed, in rich media, for measurable growth rate effects of resistance.

Materials and Methods

Strain Preparation.—BZB1011 was grown overnight in 5 ml of LB broth. All colicin-resistant mutants were derived from this same initial culture.

Colicin Preparation.—Crude colicin extracts were made of 20 Col$^+$ strains (described in Pugsley 1984; Table 1); 100 μl of an overnight culture was added to 10 ml of LB broth and grown for 2 h, at which time 0.5 mg Mitomycin C/ml (Sigma) was added. Mitomycin C causes DNA damage, stimulating the SOS system and triggering colicin production (Pugsley 1984). Eight hours later, cells were killed with chloroform and centrifuged to remove cell debris. The resulting supernatants were stored at −70°C and used for all experiments.

Selection of Resistant Mutants.—One hundred microliters of BZB1011 (approximately 4×10^8 cells) was plated on LB that had spread on it 100 μl of one of the following 11 colicins: A, D, E1–E8, and K. The aliquots of BZB1011 were from the same overnight culture, which could result in underestimating the amount of possible variation (i.e., a "jackpot effect"). Although we cannot rule out a jackpot effect, the variability observed in this study suggests that even with this selection scheme, considerable phenotypic variation could be generated.

Table 1. Colicin-bearing strains used in this study (from Pugsley 1985).

Strain	Colicin type produced
BZB2101	A
BZB2102	B
BZB2103	D
BZB2104	E1
BZB2105	E2
BZB2106	E3
BZB2107	E4
BZB2108	E5
BZB2109	E6
BZB2110	E7
PAP247	E8
BZB1407	E9
CA46	G
CA58	H
BZB2114	Ia
BZB2115	Ib
BZB2116	K
PAP1	M
BZB2101	N
PAP2	S4

Colicins were diluted to an amount that would select resistant mutants at a frequency of 10^{-6} mutants/wild type cells. Given that this frequency is similar to frequencies observed after exposure to traditional antibiotics, which typically results from single mutations (Miller 1992), these mutations are most likely single mutations. For each selective regime, 20 colonies were picked, plated again on LB, and then plated once more on LB plates supplemented with the same amount of colicin extract as during the initial selection. A single colony was chosen, grown overnight in LB, and stored in 15% glycerol at −70°C for further use. Only 19 mutants from the colicin E7 treatment were used, because one mutant became contaminated during the course of the experiment.

Resistance Assays.—One hundred microliters of an overnight culture of cells was added to 3 ml of LB top agar (0.7% Bacto-Agar) and poured on an LB plate (1.6% Bacto-Agar). Two milliliters of each colicin extract was then spotted onto this LB top agar lawn. Any clearing was scored as sensitivity, whereas absence of clearing was scored as resistance. As a control, extracts were spotted onto a lawn of BZB1011, a colicin-sensitive K-12 derivative (described in Pugsley and Oudega 1987).

Growth Rate Assays.—Strains were taken from storage at −70°C and grown for 12 h at 37°C in 10 ml of LB in 50-ml Erlenmeyer flasks at 225 rpm. After precisely 12 h, 100 μl of a 10^{-3} dilution of the overnight culture was added to 9.9 ml of LB liquid medium. At 3, 4, and 5 hours after inoculation, dilution plating on LB medium was used to quantify cell density. After plating, the plates were incubated for 24 h and enumerated.

The growth rate technique used here allows more precision than the traditional standard growth assay, which inoculates cells at 1% of carrying capacity. Pilot studies suggest that cells do not leave stationary phase (and lag phase) in fresh medium for approximately 1.5–2.5 h: data from these time

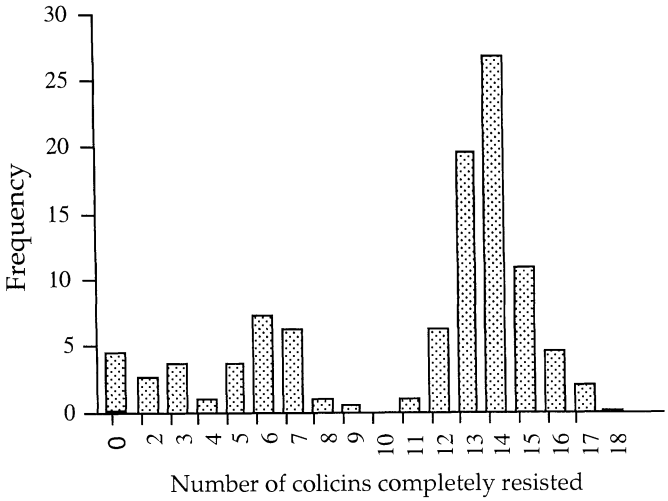

FIG. 1. The frequency distribution of the number of colicins resisted per mutant strain.

points may thus result in inaccurate readings. Pilot studies have also indicated that nutrient depletion can influence growth rates at extremely low cell densities, which begin to occur in the assay used here at approximately 6 h. Measurements were therefore made at 3–5 h of growth.

The low starting cell densities reduce the conflating effects of carrying capacity. Nutrient depletion can begin to influence accurate growth rate readings at cell densities as low as 10^6 cells/ml, leading to an underestimation of growth rate. Growth curves started at a cell density of 1% of carrying capacity (in LB, approximately 4×10^7 cells/ml) will result in depressed growth rates due to nutrient limitation. Pilot studies indicated that there was less variability associated with measurements made at very low densities.

Calculation of Growth Rate.—Cell densities at each time point were log transformed and regressed against time. The slope yielded the number of cell doublings per hour. Due to the high number of strains (61) and replicates (three for most strains), growth curves could not all be performed on one day. To control for time effects, the growth curves from each day were adjusted by assigning the growth rate of the BZB1011 control a value of one. Those mutants with a growth rate greater than one grew faster than the control, and those with a growth rate less than one grew slower than the control.

Statistical Analysis.—Analyses were performed using JMP 3.1.5 Statistical Discovery Software (SAS Institute 1996).

RESULTS

A total of 219 mutants were selected by exposure to one of 11 different colicin extracts (A, D, E1–E8, and K). The mutants selected were characterized for resistance to 20 colicins (Table 1). Ninety-six percent of the 219 mutants examined were resistant to two or more colicins, where resistance is defined as the absence of any visible inhibition (killing). This criterion was used to prevent observer bias in scoring plaques weaker than the BZB1011 control. The remaining 4% of the mutants were not resistant to any colicins (Fig. 1). These "non-resistant" mutants exhibited faint plaques; thus these mutants were not scored as resistant. However, these plaques were considerably fainter than the BZB1011 control, suggesting that some level of increased resistance had been selected.

TABLE 2. The number of different resistance profiles per colicin selection regime.

Colicin exposure	Number of profiles
A	12
D	9
E1	7
E2	6
E3	13
E4	8
E5	10
E6	12
E7	14
E8	7
K	9

Of the 219 mutants, 194 could be definitively scored for all 20 colicins, because 25 mutants had variable phenotypes for one or more colicins. Among these 194 mutants, the mean number of colicins resisted per mutant was 11.0 ± 3.6 (SD) of the 20 colicins assayed. No mutants were observed that were resistant to only one colicin.

Each mutant was assigned a resistance profile based on its pattern of resistance to the 20 different colicins (e.g., resistance to A, sensitive to B, etc.). Among the 219 mutants, there were 95 distinct resistance profiles. Table 2 provides the frequency distribution of the number of resistance profiles per colicin selection regime. Different colicin selection regimes varied widely in the number of resistance profiles generated (Table 2), ranging from six for the colicin E2 mutants (of 20 mutants scored) to 14 for the 19 colicin E7 mutants. Table 3 provides a summary of the number of mutants, regardless of selection regime, that shared a resistance profile. Seven of these 10 profiles were composed exclusively of colicin E exposed mutants. Two others were exposed to colicins that utilize the TolQ pathway (A and E1; E1, E3, E5, E6, and K). Interestingly, one colicin D resistant mutant shared a resistance profile with eight other colicin E5 resistant mutants (Table 3).

Within each selection regime, the mutants demonstrated remarkably different patterns of resistance to the 20 scored colicins (Table 4). Mutants exposed to colicin D were the

TABLE 3. Distribution of shared resistance profiles. The left column indicates which colicin selection regimes resulted in mutants with identical resistance profiles.

Colicin selection regime	Number of strains
E1, E6	4
E6, E8	2
E3, E6, E8	3
E3, E6	7
E3, E5, E7, E8	8
E2, E3, E5, E6	26
E2, E5	2
A, E1	3
E1, E3, E5, E6, K	18
D, E5	9

TABLE 4. The percentage of strains, by colicin selection regime, that are resistant to a given colicin type. DOC^r and DOC^s refer to the frequency of colicin resistance among DOC resistant and DOC sensitive mutants (colicin D exposed mutants are excluded from these two columns).

Colicin resisted	Selection regime													
	A	*D*	*E1*	*E2*	*E3*	*E4*	*E5*	*E6*	*E7*	*E8*	*K*	*all*	DOC^r	DOC^s
A	50	0	0	0	5	0	5	0	0	35	5	9	8.8	15.8
B	0	70	0	0	0	0	0	0	0	0	0	6	0	0
D	0	55	0	0	0	0	0	0	0	0	0	5	0	0
E1	25	0	65	0	55	0	0	75	60	60	0	33	34.4	35.1
E2	10	0	10	0	10	60	0	35	45	55	5	21	25.5	15.8
E3	10	0	20	85	90	100	60	80	90	95	5	58	75.2	15.8
E4	10	0	0	0	5	0	5	15	0	10	5	5	2.5	15.8
E5	10	0	20	0	20	100	5	40	75	60	5	31	37.9	15.8
E6	10	0	20	70	90	100	50	85	85	90	5	55	71.4	15.8
E7	10	0	0	0	10	70	5	20	55	60	5	22	26.6	15.8
E8	10	0	20	0	25	100	5	40	65	60	5	31	38.0	15.8
E9	65	25	90	75	95	100	45	95	95	100	100	82	86.9	89.2
G	100	55	100	100	100	95	80	95	95	100	90	93	96.9	94.7
H	100	20	100	90	100	95	40	90	75	100	100	83	87.5	100
Ia	95	0	20	0	10	100	0	5	50	45	0	30	34.8	27.0
Ib	75	0	20	0	40	90	5	20	65	35	30	36	40.8	32.4
K	10	0	0	0	0	0	0	0	0	0	5	1	0.6	5.3
M	0	30	0	0	0	0	0	5	0	0	0	15	0.6	0
N	75	0	0	0	0	0	0	0	0	10	20	10	7.5	23.7
S4	5	0	0	0	0	0	5	0	0	5	35	5	0.6	25.0

most distinct, with the highest frequency of resistance to colicins B (70%), D (55%), and M (30%). This is not surprising because colicins B and D share the FepA receptor and colicins B, D, and M use the TonB translocation pathway (Pugsley 1984). Mutants resistant to colicin A were also quite distinct phenotypically, with low frequencies of resistance to most colicins with the exception of resistance to colicins A, E9, G, H, Ia, Ib and N. Colicin K mutants had low frequencies of resistance to most colicins, including colicin K, with the exceptions of colicins G, H, Ia, and Ib. The colicin E resistance mutants were themselves variable. Those mutants exposed to colicins E3, E4, E6, and E8 were resistant at high frequencies to most of the E colicins and colicins G, H, Ia, and Ib. The other colicin E resistance mutants possessed lower frequencies of resistance to the E colicins. It is unclear how exposure to the A, E, and K colicins would result consistently in resistance to colicins G, H, Ia, and Ib, because the E colicins utilize different receptors and translocation pathways than the G, H, Ia, and Ib colicins (Pugsley 1984).

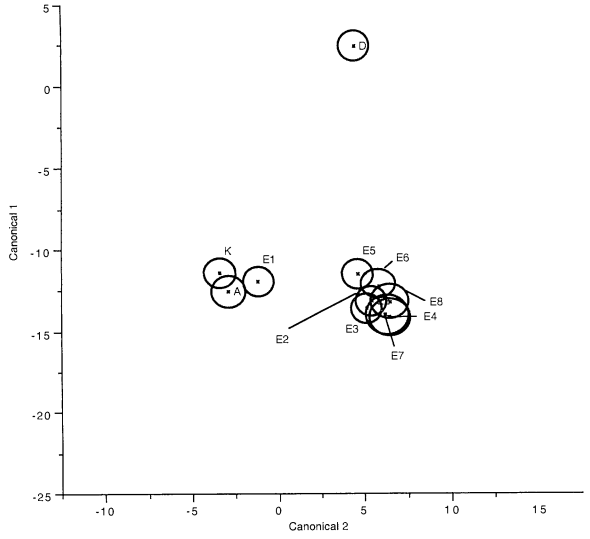

FIG. 2. Canonical hierarchy analysis of the phenotypes of the resistance mutants by selection regime. The circles correspond to the 95% confidence region for each group of mutants, with the x marking the center of distribution.

One interesting pattern was the occasional sensitivity to the colicins to which mutants had been originally exposed. These mutants exhibited very faint plaques when spotted with the colicin that was used to select for resistance. However, because all mutants were selected at frequencies of 10^{-6} or lower, we believe that this level of resistance is sufficient for protection against colicin killing. Thus, our criterion for resistance (no observable inhibition) might be conservative, although it is a reliable criterion that is not subject to observer bias.

Resistance profiles were subjected to canonical analysis (Dillon and Goldstein 1984) to determine if mutants selected for resistance to different colicins were phenotypically distinct. The mutants, analyzed on the basis of resistance to 20 colicins, were divided into classes based on their selection regime. Essentially, in this sort of analysis an abstract, two-dimensional phenotype space is constructed and the strains are placed on this landscape on the basis of their resistance profiles (Fig. 2). Colicin D mutants were extremely different from all other mutants, because they were the only mutants that resisted colicins B and D at high frequencies (Table 4). Mutants selected on colicins A, E1, and K were very similar to each other and very different from the other E colicins. Colicin A and K selected mutants were not distinct from each other, whereas E1 selected mutants were distinct from A and K selected mutants. The majority of the E colicin mutants were similar to each other, except for the colicin E5 selected mutants, which were similar only to the colicin E6 mutants

TABLE 5. The number of different colicin selection regimes that led to resistance to a given colicin.

Colicin resisted	Number of different exposures that led to complete resistance
A	5 (A, E3, E5, E8, K)
B	1 (D)
D	1 (D)
E1	6 (A, E1, E3, E6–E8)
E2	8 (A, E1, E3, E4, E6–E8, K)
E3	10 (A, E1–E8, D)
E4	6 (A, E3, E5, E6, E8, K)
E5	9 (A, E1, E3–E8, K)
E6	10 (A, E1–E8, K)
E7	8 (A, E3–E8, K)
E8	9 (A, E1, E3–E8, K)
E9	10 (A, E1–E8, K)
G	10 (A, E1–E8, K)
H	10 (A, E1–E8, K)
Ia	7 (A, E1, E3, E4, E6–E8)
Ib	9 (A, E1, E3–E8, K)
K	2 (A, K)
M	2 (D, E6)
N	3 (A, E8, K)
S4	4 (A, E5, E8, K)

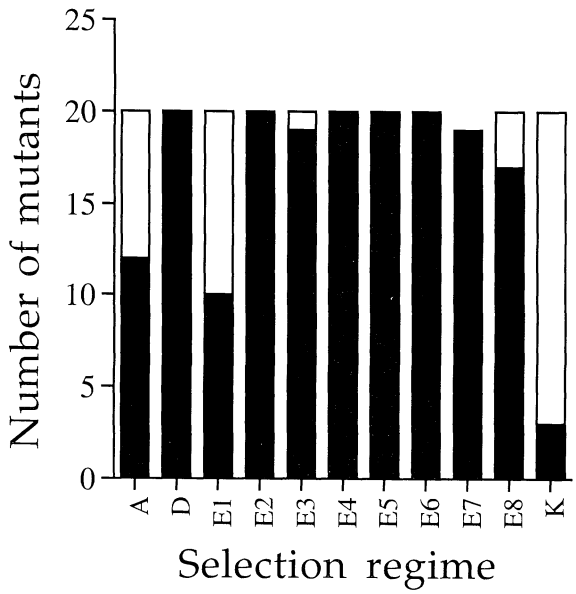

FIG. 3. The number of putative resistance (black shading) and tolerance mutations (white bar) by selective regime.

and distinct from the other E colicin selected mutants (Fig. 2).

Within the entire collection of resistance mutants, resistance to colicins E9, G, and H occurred at the highest frequencies (Table 4), even though most of the colicins used to select mutants do not share uptake mechanisms with colicins G and H. Resistance to E9 (82%) was higher than any of the other E colicins (5–55%). Colicin E4 resistance was the least commonly generated resistance among the E colicins (5%). The frequency of resistance to colicins B, D, and M was very low, as would be expected because only 20 of 219 mutants were exposed to colicins that used either the FepA receptor or the TonB pathway.

In general, many different colicin exposures gave rise to the same resistance profiles. Not unexpectedly, those colicin resistances that were selected infrequently were derived from fewer different colicin exposures (Table 5). One exception was resistance to colicin A. Although resistance to colicin A was generated infrequently, it was selected from five different colicin exposures (A, E3, E5, E8, and K). Resistance to colicins B and D was selected only after exposure to colicin D, which would be predicted because colicins B and D do not share receptors and translocation pathways with the other colicins used to select for resistance mutants (A, E1–E9, K, N, and S4).

The Effects of Mutational Class

Although genetic mapping would be required to definitively determine the locus or loci involved in the generation of resistance, phenotypic techniques can be used to subdivide the mutants into broad mutational classes. Colicin resistance often results in phenotypic changes not directly related to colicinogeny or colicin resistance. For example, all characterized Tol pathway mutations also confer sensitivity to 1% sodium deoxycholate (DOC; Webster 1991). Those mutants that were sensitive to DOC were classified as translocation (Tol pathway) mutants, whereas those that were resistant to DOC were classified as putative receptor mutants. Each of the 219 mutants in this study were screened for resistance to DOC (Bernstein et al. 1972). Seventeen of 20 mutants selected for resistance to colicin K were sensitive to DOC, indicating that the mutations conferring resistance in this case occurred in the Tol pathway (Fig. 3). The mutants that were selected for resistance to colicin E1 were evenly split between putative Tol and BtuB mutations. Colicin E1 selection is known to result in a high frequency of *tolC* mutants (Whitney 1971). Of the 159 mutants selected on colicins E2–E8, 155 were resistant to DOC, suggesting that the overwhelming majority of these mutants (97.5%) possessed *btuB* mutations. All colicin D mutants were resistant to DOC, as would be expected because resistance to colicin D does not involve alterations of the Tol pathway (Fig. 4).

The Tol mutants were less resistant to most colicins, with the exceptions of colicins A, K, N, and S4 (Table 4). The higher frequencies of resistance to colicins A, K, N, and S4 among the putative Tol mutants is expected because some Tol mutations are believed to confer resistance to these colicins. Likewise, very few of the DOC^r mutants (receptor mutants) were resistant to colicins A, K, N, and S4 because these colicins use different receptors than the E colicins. One pattern that is difficult to explain is the frequency of resistance to the I colicins. DOC^r mutants were more likely to be resistant to the I colicins than were the DOC^s mutants (sensitive mutants). How putative receptor mutations (*btuB* or *ompF*) could alter I colicin killing is unknown.

Does Resistance Impose a Fitness Cost?

Maximal growth rate was used as an assay for fitness. Because the ability to monopolize a resource rapidly is of significant importance in situations where resources are not continuously replenished (Vasi et al. 1994), in many micro-

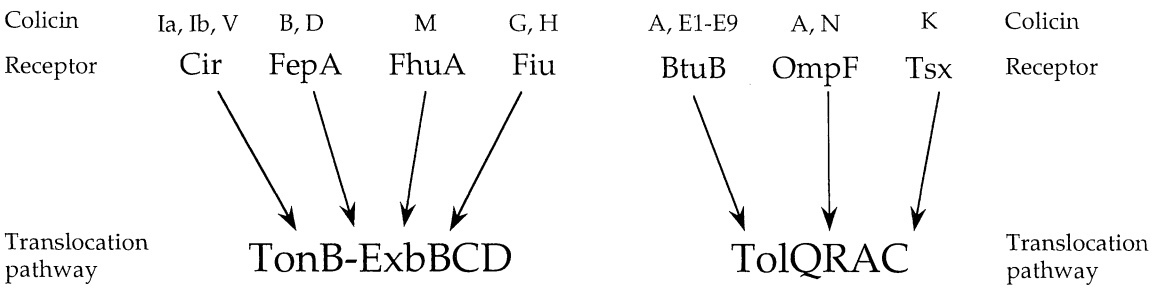

FIG. 4. A schematic of the proteins involved in colicin binding and uptake into the bacterial cell. The top row indicates the colicin type, with those colicins used to generate mutants in bold face. The next row indicates the receptor protein used and the bottom row indicates the translocation pathway.

bial environments maximal growth rate may approximate fitness. Additionally, all mutants reached the same density in stationary phase; thus, density at stationary phase was not used to estimate fitness.

The average doubling time in rich medium of BZB1011, the sensitive strain from which the mutants were derived, was 1.56 doublings/h. The mean adjusted maximal growth rate of the mutant strains was 0.95 (SD = 0.14; $n = 60$, $t = -3.901$, $P = 0.0001$), which was significantly lower than the growth rate of BZB1011, (assigned a growth rate of 1.0). This growth difference means that under conditions of continuous growth, if a sensitive and an average resistant strain were inoculated at equal frequencies in rich media, the sensitive strain would outnumber the resistant strain by four orders of magnitude after 190 h. This displacement of resistance by sensitivity is not as rapid as the displacement of sensitivity by colicin production, which can occur in as few as 12 h (Chao and Levin 1981; Tan and Riley 1996). However, a 5% difference in growth rate is still a significant growth rate advantage for sensitive strains.

When the growth rates of resistant mutants are compared individually to BZB1011, 23% of the isolates (13/61) grew significantly ($P < 0.01$) slower than BZB1011. Many additional strains had lower mean growth rates than BZB1011, but were not statistically distinguishable from it ($P > 0.05$). Of the 14 strains with significantly depressed growth rates, four had been selected for resistance to colicin E1 and 10 for resistance to colicin K. One mutant selected on colicin E5 grew faster than BZB1011, with a growth rate of 1.03 ($P = 0.0001$).

ANOVA indicated that colicin exposure significantly affected growth rate, whereas the effects of strain and date of measurement were insignificant (Table 6). Only those mutants selected for resistance to colicins E1 and K had growth rates significantly lower than BZB1011 (Table 7). The mean growth rate of the colicin E1 selected mutants was 0.87 ± 0.16 (SD), and the mean growth rate of the colicin K selected mutants was 0.84 ± 0.09 (SD).

Among the 60 mutants assayed for growth rate, there were 20 shared resistance profiles. In the majority of cases, strains with identical colicin resistance phenotypes did not differ significantly in fitness. Two of the colicin K selected strains possessed significantly distinct growth rates, and both of these strains grew slower than the sensitive control. Of the 20 resistance profiles included in the growth rate study, only two were composed of strains that exhibited different growth rates. One profile with two colicin E1 selected mutants (both putative receptor mutants) had strains with significantly different growth rates ($P < 0.0001$). The other resistance profile of strains with variable growth was composed of six strains that were resistant to only colicins E9, G, and H.

Overall, Tol mutations appear to be costly, whereas receptor mutations do not appear to be costly. DOC resistant strains (putative receptor mutants) did not grow slower (0.99 ± 0.11, $n = 32$, $P = 0.878$) than the sensitive ancestor, while DOC sensitive strains (putative Tol mutants) grew significantly slower (0.83 ± 0.12, $n = 18$, $P < 0.0001$) than the sensitive control. A greater frequency of Tol strains grew significantly slower than the putative receptor mutants (52.6% vs. 12.5%, $G_1 = 9.1503$, $P < 0.005$).

DISCUSSION

Surveys of natural populations and a wealth of molecular and genetic data suggest that multiple colicin resistances can be selected by exposure to a single colicin. To determine if this form of pleiotropy is a significant factor in the generation of resistance phenotypes, 219 mutants were selected for resistance to one of 11 colicins. The 11 colicins chosen for the

TABLE 6. ANOVA of growth rates. Colicin exposure is the colicin to which a mutant was exposed. Mutant represents the effect of each individual mutant. Replicate tests for date of measurement effects. All sources were treated as random effects ($R^2 = 0.98$).

Source	df	MS	F	P
Colicin exposure	5	0.1727	12.542	< 0.0001
Mutant (exposure)	56	0.0208	1.054	0.4299
Replicate (mutant, exposure)	108	0.014	2.095	0.0985
Error	10	0.0067		

TABLE 7. The mean growth rate of mutants selected for resistance to different colicins.

Selection regime	Mean growth rate	Standard deviation	P
A ($n = 10$)	0.9645	0.1612	ns
D ($n = 10$)	1.0390	0.1408	ns
E1 ($n = 10$)	0.8762	0.1603	0.0039
E4 ($n = 10$)	0.9848	0.0840	ns
E5 ($n = 10$)	0.9975	0.1064	ns
K ($n = 10$)	0.8406	0.0888	< 0.0001

resistance selection represent a range of different killing mechanisms.

Colicins A, D, E1, and K are pore-forming colicins, whereas colicins E2, E7, and E8 act as DNases and colicins E3, E6, and E5 have RNase activity (Pugsley 1984; James et al. 1996). With the exception of colicin D, all of these colicins share the same translocation pathway, the TolQ pathway (Pugsley 1984; Fig. 4). Additionally, the E colicins and colicin A share the BtuB receptor (Pugsley 1984; James et al. 1996; Fig. 4). Due to the shared receptors and translocation pathways, it was expected that multiple resistance would be selected following exposure to a single colicin. However, despite their common binding and uptake mechanisms, exposure to different colicins might yield different resistance profiles in the selected mutants. The E colicins may bind to slightly different regions of the BtuB receptor (Smarda and Sevcikova 1988). In addition, colicin E1 appears to use only the TolQ pathway in a unique manner; thus, any translocation mutations resulting from exposure to colicin E1 may possess different phenotypic effects than those exposed to the other E colicins (Schendel et al. 1997).

Ninety-six percent of the mutants exhibited increased resistance to two or more colicins, indicating that multiple resistance is ubiquitous. Thirty-seven percent of the isolates shared a resistance profile with a mutant that was selected for resistance to a different colicin, indicating that different colicin selection regimes can result in the same multiple resistance phenotype. Pleiotropy in these instances results in identical phenotypes despite different selective pressures.

When all mutants are considered, there is no significant correlation between the frequency of resistance in natural populations and the frequency of resistance among the mutants selected here. However, 73% of the mutants were selected for resistance to one of the E colicins, which could bias the mutant resistance frequencies toward resistance to the E colicins in our study. Additionally, selection for resistance to the E colicins should not result in resistance to those colicins that use a different translocation pathway (Davies and Reeves 1975a,b). Thus, only the frequencies of the mutants generated from E colicin exposures will be related to the frequencies of colicin resistance in natural populations discussed below.

When resistance among the mutants is scored as any deviation in phenotype from the sensitive strain, a highly significant positive correlation is found between resistance to the E colicins among the mutants and natural isolates (Fig. 5; $P = 0.0003$; $R^2 = 0.86$). In other words, the frequency of resistance in natural populations is strongly correlated with the frequency of resistance among the mutants. This suggests that the frequencies of colicin resistance in natural populations might be partly influenced by the molecular pathways of resistance. Hence, the scarcity of colicin E4 resistance in natural populations might be in part due to the rare generation of resistance to colicin E4. Thus, pleiotropy may limit the possible resistance phenotypes available to *E. coli*.

It has been assumed that colicin resistance imposes a fitness cost, by analogy to other types of resistance including antibiotic resistance and phage resistance (Zund and Lebek 1980; Lenski 1988a; Cohan et al. 1994). Our data suggest that the assumption of antagonistic pleiotropy, involving a trade-off between fitness and resistance, is not always appropriate. Overall, there is a significant cost associated with colicin resistance ($s \simeq 0.05$/h). These costs are comparable to the cost of antibiotic resistance (Dykhuizen and Hartl 1983), but are three to five times less than the cost of phage T4 resistance (Lenski 1988a). However, not all types of phage resistance (e.g., phage T5; Birge 1994) confer lower growth rates, and here too in our mutant survey, cost-free mutants are observed.

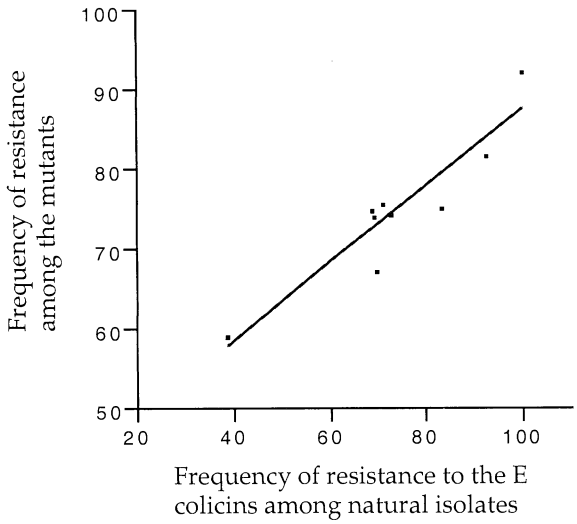

FIG. 5. The correlation between the frequency of resistance to the E colicins among the mutants (excluding the D selected mutants) and among the natural isolates.

Twenty-four percent of the colicin resistant mutants studied for growth rate effect were significantly less fit (i.e., grew slower) than the sensitive isogenic strain. Mutants from only two of the six colicin selection regimes (E1 and K) imposed a fitness cost. Even within these two selection regimes, however, the fitness effect of resistance was variable. For example, four colicin E1 selected mutants were less fit than the sensitive control, but six other such mutants were not. These data suggest a complex relationship between the fitness of a cell and its colicin resistance phenotype. First, there is no correlation between the number of different colicins resisted (completely or partially) and the cost of resistance. Second, although the general trend suggests a fitness cost associated with resistance, many resistance mutations are neutral under the conditions assayed here. A more detailed examination of the specific resistance mutations may help resolve these conflicting results. Receptor mutations (DOC^r) generally were neutral and conferred resistance to more colicins, whereas Tol mutations (DOC^s) were often deleterious and less likely to confer multiple resistance. Thus, certain colicin exposures are likely to select for resistance that is costly (e.g., colicins E1 and K).

The high frequency of colicin resistance observed in nature suggests either that the cost of resistance is lower than the costs measured in this study or that the pressures imposed by colicinogenic strains are significant and extended. Two general evolutionary mechanisms for the amelioration of deleterious fitness costs have been proposed. "Compensation" involves changes at other loci that reduce or eliminate the

cost of deleterious pleiotropic effects (Fisher 1928). Compensatory mutations for colicin resistance might be found in loci that encode for membrane proteins that interact either with altered receptors or altered translocation proteins. Although the existence of compensatory mutations in mitigating the deleterious side effects of colicin resistance has not been investigated, compensatory mutations can arise that decrease the cost of resistance to phage T4 (Lenski 1988b) and the cost of rifampicin resistance in *Bacillus subtilis* (Cohan et al. 1994).

"Replacement" can also eliminate the deleterious pleiotropic effects of mutation through the substitution of alleles that are less costly or neutral (Haldane 1932; Chevillon et al. 1997). In this study, 24% of colicin mutations resulted in noticeable fitness decreases, whereas others bore no measurable fitness cost. Resistance mutations that lack the fitness costs associated with resistance but confer continued resistance to colicins might be prevalent in natural populations.

The role of colicinogeny in natural environments is unclear. In both laboratory and theoretical models, certain conditions allow colicinogenic strains to invade and displace sensitive populations (Frank 1994; Riley and Gordon 1996; Durret and Levin 1997). However, these model systems have generally not incorporated the evolution of resistance among the sensitive population in response to colicin presence. Riley and Gordon (1996) speculate that populations may cycle among sensitive, colicinogenic, and resistant states. In this scenario, when colicin-producing strains invade a sensitive population, intense selection for colicin resistance will create a population of resistant strains that potentially could displace the colicinogenic population. If resistance were costly, sensitive revertants might then arise and outcompete the resistant population, thus resetting the system to the sensitive state. This might result in the population cycling envisioned by Riley and Gordon (1996).

This scenario makes two assumptions. First, resistant strains must be able to invade colicinogenic populations. Second, once resistant strains predominate, the cost of resistance must outweigh the benefits so that sensitivity can reinvade. As has been detailed previously, there are additional beneficial consequences of colicin resistance, including resistance to multiple colicins and phage resistance (Hancock et al. 1976). There are also possible additional deleterious effects of resistance, beyond the growth rate differences in rich media reported here, such as reduced nutrient uptake (Pugsley and Reeves 1976) and increased sensitivity to antibiotics, bile salts, and detergents (Savage 1977; Mitsuoka 1996).

Pleiotropy is often assumed to act antagonistically. Our data suggest that this might not be the case, because those mutations that confer resistance to many colicins often are not costly under laboratory conditions. Additionally, given the high frequency of colicin-producing strains, even deleterious mutations could be retained because selection for a resistance to a different colicin, even if not the original selective force, could maintain a particular mutation. Considering the high levels of colicin resistance among natural isolates of *E. coli*, it would appear that the benefits of colicin resistance in most cases outweigh the potential costs of resistance. Nonetheless, colicin-sensitive strains can be isolated. This study examined strains isolated from fecal material. Colicin sensitivity might be favored in harsher, nutrient-limited habitats (e.g., soil or aquatic habitats) and thus be more common in these habitats. If there is a significant division between *E. coli* that primarily inhabit the gut and those that only infrequently inhabit the gut, as suggested by Savageau (1983), the pleiotropic consequences of resistance might be a factor in this ecotypic subdivision and we would predict lower levels of colicin resistance in these nongut populations. Thus, the effects of enviromentally contingent pleiotropy may lead to ecological divergence in different habitats.

Acknowledgments

The authors would like to thank P. Fu, N. Okolo, and M. Santos for their assistance. This work was funded by grants from the National Institutes of Health (First Award), National Science Foundation (Young Investigatory Award), and General Reinsurance Corp. awarded to MAR.

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Corresponding Editor: W. T. Starmer