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RESEARCH ARTICLE

The Evolution of Reduced Microbial Killing

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Bacteria engage in a never-ending arms race in which they compete for limited resources and niche space. The outcome of this intense interaction is the evolution of a powerful arsenal of biological weapons. Perhaps the most studied of these are colicins, plasmid-based toxins produced by and active against *Escherichia coli*. The present study was designed to explore the molecular responses of a colicin-producing strain during serial transfer evolution. What evolutionary changes occur when colicins are produced with no target present? Can killing ability be maintained in the absence of a target? To address these, and other, questions, colicinogenic strains and a noncolicinogenic ancestor were evolved for 253 generations. Samples were taken throughout the experiment and tested for killing ability. By the 38th transfer, a decreased killing ability and an increase in fitness were observed in the colicin-producing strains. Surprisingly, DNA sequence determination of the colicin plasmids revealed no changes in plasmid sequences. However, a set of chromosomally encoded loci experienced changes in gene expression that were positively associated with the reduction in killing. The most significant expression changes were observed in DNA repair genes (which were downregulated in the evolved strains), Mg ion uptake genes (which were upregulated), and late prophage genes (which were upregulated). These results indicate a fine-tuned response to the evolutionary pressures of colicin production, with far more genes involved than had been anticipated.

Introduction

Bacteria engage in a never-ending arms race in which they compete for limited resources and niche space. The outcome of this intense interaction is the evolution of a diverse and powerful arsenal of biological weapons. Most species of bacteria produce one, and usually many more, of these potent biocontrol agents, including classical antibiotics, lytic agents, lysozymes, and bacteriocins (Cascales et al. 2007). One predictable outcome of the use of biological weapons is the rapid response from the target community, resulting in the evolution of resistance mechanisms. A human-mediated version of this arms race has resulted from our use of broad-spectrum antibiotics, in which the target species, human pathogens, rapidly evolved resistance to the antibiotics employed in their decimation.

The best-studied bacterial arms race is mediated by colicins, which are potent toxins produced by and active against *Escherichia coli* (Cascales et al. 2007). Colicins are members of a much larger group of toxins known as bacteriocins, which are compounds produced by bacteria that inhibit or kill closely related species (James et al. 1991; Vuyst and Vandamme 1994). Their production occurs across all major groups of Eubacteria and the Archaeobacteria (Webster 1991). Despite high levels of bacteriocin diversity, these proteins share many general characteristics (James et al. 1991; Vuyst and Vandamme 1994). They are generally high-molecular weight protein antibiotics that kill closely related strains or species. The bacteriocin gains entry into the target cell by recognizing specific cell surface receptors and then kills the cell by forming ion-permeable channels in the cytoplasmic membrane, by nonspecific degradation of cellular DNA, by inhibition of protein synthesis through the specific cleavage of 16s rRNA, or by cell lysis

resulting from inhibition of peptidoglycan synthesis. Frequently, the bacteriocin is released from the cell through the action of a lysis protein, although other export mechanisms may be involved. The producing strain shows specific immunity toward its bacteriocin through the production of an immunity protein that usually interacts directly with the C-terminal domain of the bacteriocin protein.

The bacteriocin phenotype is usually encoded for by a gene cluster of three closely linked, frequently plasmid encoded, genes: the bacteriocin, immunity, and lysis genes. Nuclease colicins, like colicin E2, have a conserved operon structure in which the colicin genes (*cxa*) are the first genes in an operon that also encodes an immunity gene (*cxi*) and lysis gene (*cxl*; Cascales et al. 2007). For illustration, the organization of the colicin E2 plasmid is depicted in figure 1 that include the colicin operon (Cole et al. 1985), the regions responsible for plasmid replication, the origin of replication (Hiraga et al. 1994), and several restriction endonuclease sites (Pugsley and Schwartz 1983). The three genes in the colicin operon are cotranscribed and under LexA control (Gillor et al. 2008); however, the immunity gene is also expressed from its own constitutive promoter (Cascales et al. 2007).

Target cells can evolve resistance or tolerance to the effects of bacteriocins, generally by altering a cell surface receptor or translocation system. All of these characteristics are possessed by the well-studied bacteriocins of *E. coli*, the colicins (Pugsley 1984; Pugsley and Oudega 1987; James et al. 1991), which are the focus of this study.

In contrast to the broad-spectrum antibiotics employed by humans, colicins target only their closest relatives, which they recognize by the presence of specific cell surface receptors. Resistant strains rapidly outcompete the producers due to the high costs associated with colicin production (Feldgarden and Riley 1999). This colicin-mediated dynamic between sensitive, resistant, and producer strains has been explored in great detail using a combination of in vivo (Kirkup and Riley 2004), in silico (Gordon and Riley 1999), and in vitro (Kerr et al. 2002)

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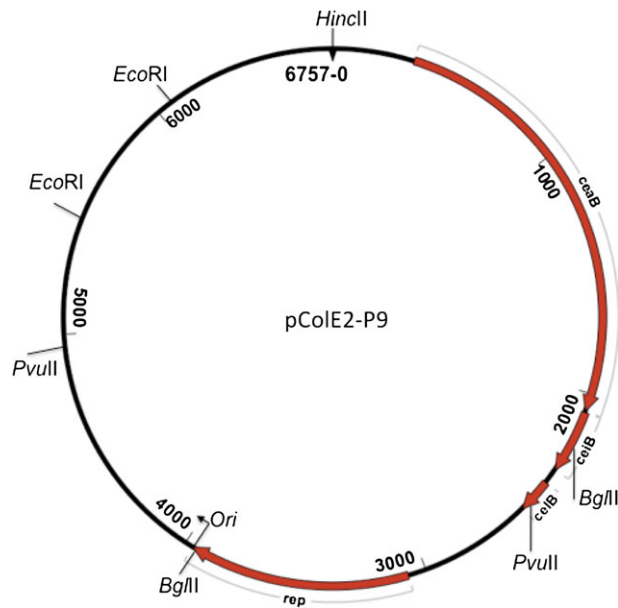


FIG. 1.—The pColE2-P9 plasmid map.

models. Pairwise in vitro competitions between these three strain types are resolved in a predictable order. In a geographically structured environment, the producer strains kill their sensitive targets, resistant strains outcompete producers, and sensitive strains outcompete resistant strains. The presence of all three strain types results in the establishment of a nontransitive, dynamic equilibrium that has been described as analogous to the childhood game of Rock-Paper-Scissors (Kerr et al. 2002).

In an earlier attempt to evaluate the importance of colicin production on *E. coli* strain residence in the gastrointestinal tract, an in vivo experimental evolution study was undertaken using a mouse model (Gillor et al. 2009). Colicin-producing *E. coli* and their nonproducing ancestors were established in the large intestine of mice, previously treated with streptomycin to clear the resident Gram-negative bacteria. The introduced *E. coli* strain's persistence, population density, and doubling time were monitored over a period of 112 days. Early in the experiment, only minor differences in population density between the various bacteriocin producing and nonproducing control strains were detected. However, over time, the persistence of the control strains plummeted, whereas the density of the colicin-producing strains remained significantly higher. In a subsequent study, also applying the mouse model, but with competition introduced (i.e., mice with different colicinogenic strains cocaged), the production of colicin E2 significantly decreased over time (Riley M and Gillor O, unpublished data).

The present study was designed to provide a more controlled environment with which to further explore the molecular responses of a colicin-producing strain exposed to extended growth (253 generations) under serial transfer conditions. The in vitro experiment shares certain features with the first mouse model described above; there is continuous culturing of the strains, there are no known direct

competitors present, and the population dynamics and levels of colicin production were followed over an extended period of time. The in vitro experiment also differs from the mouse model in several fundamental ways. Perhaps the most important differences involve the lack of a community structure in the serial transfer flasks and the homogeneous liquid environment provided. In spite of these significant differences, the killing ability of the producing strain was shown to significantly decrease over the course of the serial transfer experiment, just as was observed in the mouse study (Riley M and Gillor O, unpublished data). The impact of this reduced killing was examined at the phenotype level, using growth assays, and at the genome level, using plasmid DNA sequencing and chromosomal microarrays, to identify loci potentially involved in this evolution of reduced microbial killing.

Materials and Methods

Bacterial Strains

The ancestral strain, REL606^{ara}⁻, was chosen due to its frequent prior use in evolutionary studies (Lenski et al. 1991, 2003; Vasi et al. 1994; Cooper et al. 2003; Woods et al. 2006). It contains no plasmids or functional bacteriophage (Lenski et al. 1991), is sensitive to colicins E2 and E7 (data not shown), and resistant to streptomycin (Lederberg 1966). CVE2 and CVE7 were derived from REL606 by transformation with the pColE2 and pColE7 plasmids (Pugsley 1985). Plasmid DNA was prepared and transformed into REL606 (Sambrook et al. 1989). The resulting CVE2 and CVE7 strains were verified by assaying colicin production.

Experimental Evolution Growth Conditions

Three replicate populations of REL606 and CVE2 were grown in 50-ml flasks with 10-ml Luria Broth (LB) and streptomycin (100 µg/ml) for 12 h at 37 °C with rotation at 220 rpm. 100 µl of culture (3×10^8 and 2×10^8 for REL606 and CVE2, respectively) was transferred into fresh LB media to inoculate the next round of serial transfer. An 800-µl aliquot of culture was sampled prior to transfers 4, 8, 11, 15, 19, 23, 31, and 38, mixed with 200-µl glycerol, and frozen. A screen to identify potential contamination involved plating dilutions from each population on McConkey and LB plates supplemented with 10 g/l arabinose and tetrazoleum at transfers 8, 15, 23, and 38 (Levin et al. 1977). Colonies of REL606 and CVE2 appear red in the presence of arabinose and tetrazoleum, due to a mutation at the arabinose operon, whereas most *E. coli* are *ara*⁺ and produce a white phenotype (Lenski et al. 1991). No external contaminants were identified over the duration of the experiment.

Colicin Killing Assays

The samples collected from the serial transfer experiments were used to inoculate 5-ml LB with streptomycin (100 µg/ml) and grown overnight at 37 °C. Overnight cultures of REL606 or CVE7 were used to prepare lawns on LB plates, and 1 µl from each serial transfer sample was

spotted onto each lawn. Three replicate plates were spotted with three replicate spots for each of the serial transfer samples. After overnight growth at 37 °C, the diameter of each inhibition zone was measured twice and the average taken (Richardson et al. 1968; Yang et al. 2007). 1 ml from each of the overnight cultures was centrifuged at 5,000 rpm for 2 min to pellet the cells. The pellets were washed twice in LB, resuspended in 1 ml LB, held on ice, and spotted onto REL606 and CVE7 lawns as described above. 100 µl chloroform was added to 1 ml of the overnight cultures described above and vortexed. The culture was centrifuged at 13,000 rpm for 10 min. This spent media was collected in a sterile eppendorf tube, held on ice, and spotted onto REL606 and CVE7 lawns as described above.

Growth Assays

Relative fitness was expressed as maximum growth rate (V_{max} ; Vasi et al. 1994). Overnight cultures in LB were prepared from the frozen aliquots and used to inoculate three 50 ml flasks with 10 ml LB. Cultures were grown at 37 °C, with shaking at 220 rpm. At ~30 min intervals, the optical density (OD) was determined. The OD readings were plotted on a log scale and a regression line calculated. The slope of this regression line provides a measure of the instantaneous growth rate constant (α). The V_{max} values were determined using three time points for each growing culture. The relative V_{max} was calculated by dividing these sample measures by the average V_{max} of the ancestral strain. A two-sided Welch approximate t -test was employed to determine significant differences in maximal growth rate between the ancestral and evolved strains, as described in (Cooper et al. 2001).

pColE2 Sequence Determination

The DNA sequences of the pColE2 ancestral and evolved plasmids were determined by primer walking, using the existing sequence data for the colicin E2 gene cluster as the starting point (Cole et al. 1985). DNA sequencing was performed according to BigDye Terminator v3.1 Cycle Sequencing Kit manual. Samples were analyzed using a 3100 Genetic analyzer (Applied Biosystems). Analysis of the resulting DNA sequences was performed using four peaks (Neefix laboratories) and the Laser gene suite (DNASTAR).

Transcriptional Profiling

Two samples of REL606 and CVE2 were obtained for each of two growth conditions (exponential and stationary phase) at transfers 1, 4, and 38 of the serial transfer experiment. Microarray samples were obtained by inoculation of 25 ml LB with 250 µl from an overnight culture. The cells were grown at 37 °C and 220 rpm to an OD = ~0.5 (exponential phase) and OD = ~1.5 (stationary phase). When the appropriate OD was reached, 10 ml of culture was transferred to a 10 ml conical tube and spun at 1660 g for 8 min (Beckman Allegra 6 Centrifuge). Pellets were resuspended in 500 µl LB, and spun at 2660 g for 1 min, and the super-

natant removed. The residual cells were flash frozen and stored at -80 °C. RNA isolation, hybridization, and detection of Affymetrix genome 2 arrays were performed according to the manufacturers protocols (Affymetrix, 2004) at Rutgers's Bionomics Research and Technology Center (BRTC, EOHSI Building, Piscataway, NJ). Affymetrix GeneChip *E. coli* genome 2 arrays were employed according to the instructions of the manufacturer. The GeneChip *E. coli* genome 2.0 array tiles probe sets include all open reading frames and over 700 intergenic regions (IRs), for a total of 10,112 "loci" from four *E. coli* genomes: K12 (Blattner et al. 1997; Riley et al. 2006), O157:H7-EDL933 (Perna et al. 2001), O157:H7-SAKAI (Hayashi et al. 2001), and CFT073 (Welch et al. 2002) (www.affymetrix.com/Products_services/arrays/specific/ecoli2.affx). Locus tags start with the character "b," "z," "Ecs" or "c," for loci in K12, O157:H7-EDL933, O157:H7-SAKAI, and strain CFT073, respectively. Hybridization intensity was measured for each locus and intensity distributions generated. Because these distributions are not standard normal, the median hybridization value was used. Hybridization data were analyzed using the .CEL files in ArrayAssist 5.5.1 (Stratagene) with the following data transformations: 1) robust microarray averaging was chosen because it outperforms many algorithms in spike-in studies (ArrayAssist manual 2006), 2) log₂ transformation was employed to normalize the data to allow standard statistical analysis, 3) differential expression analysis was performed using the unpaired t -test, with asymptotic probability calculations. No corrections were employed because they tend to be too conservative (ArrayAssist manual 2006, Rice 1989), and 4) identification of upregulated and downregulated loci was performed. Probe sets were considered differentially expressed when expression levels differed significantly with $\alpha = 0.05$ and a ≥ 1.5 -fold change (Guisbert et al. 2007).

Identification of COGs

The Blattner gene annotations of the K12 genome (Blattner et al. 1997) and the databases created by Riley et al. (2006) were employed to identify clusters of orthologous genes (COGs). Synonyms for gene names were identified in Riley et al. (2006) and ecogene (Rudd 2000). The functional categories applied were from the National Center for Biotechnology Information COG database (Tatusov et al. 2003).

Results

Evolution of Reduced Killing

A colicin E2-producing derivative of REL606 Δara (CVE2) was subjected to experimental evolution in serial transfer culture for a total of 38 transfers, which corresponds to approximately 253 generations. Figure 2 provides estimates of CVE2 killing ability over time, measured as the diameter of the killing zone on the colicin E2 sensitive REL606 Δara ancestral strain. The kill zone decreased significantly (9%) in CVE2 over the course of the serial transfer experiment ($P = 5.5 \times 10^{-9}$). Even larger decreases are

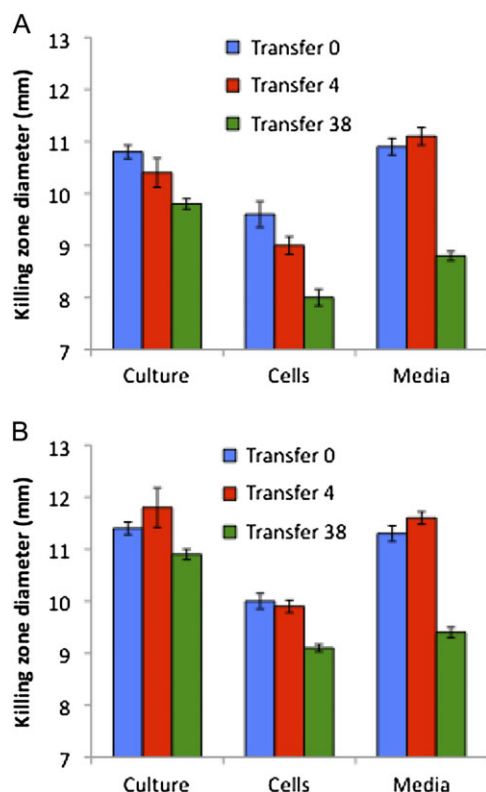


FIG. 2.—CVE2 kill zones on lawns of REL606 (A) and CVE7 (B).

observed when live CVE2 cells from the culture are assayed alone (17%) and when the cell-free spent media is examined (19%; $P = 2.7 \times 10^{-7}$ and 3.9×10^{-9} , respectively; fig. 2).

Figure 2 also provides kill zone measurements for CVE2 assayed on a strain derived from the ancestral REL606 strain by the introduction of the colicin E7 plasmid (pColE7), resulting in strain CVE7. The kill zone decreased significantly (4%) on CVE7 over the course of the experiment ($P = 1.1 \times 10^{-3}$). And, again, even larger decreases were observed when live CVE2 cells alone (9%) or their cell-free spent media (17%) were assayed on CVE7 ($P = 1.5 \times 10^{-20}$ and $P = 1.4 \times 10^{-18}$, respectively). The CVE2 kill zones measured on the CVE7 lawn are significantly larger than those observed on the REL606 *ara* lawn ($P = 5.1 \times 10^{-13}$; fig. 2).

Evolution of Growth Rate

The maximum growth rate (V_{max}) of ancestral REL606 (1.35 ± 0.02 generations per hour) and CVE2 (1.27 ± 0.01) differed significantly ($P < 0.01$). The fitness of both strains increased significantly over the 38 transfers (12% and 10% for REL606 and CVE2, respectively, $P < 0.01$, fig. 3). After the fourth transfer, however, CVE2 experienced a transient fitness decrease relative to both REL606 and the ancestral CVE2 ($P < 0.01$, in both cases; fig. 3).

pColE2 Sequence Determination

To determine if the reduced killing was due to changes in the colicin-encoding plasmid, the DNA sequences were

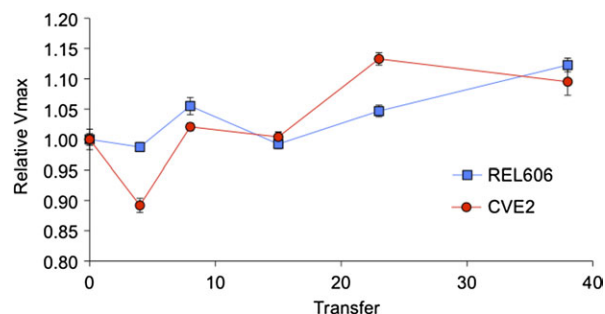


FIG. 3.—Relative V_{max} of ancestral and evolved REL606 and CVE2. Values expressed are relative to the ancestral REL606 or CVE2, respectively.

determined for the ancestral pColE2 in CVE2 and for one sample from each of three replicate cultures at $T = 38$. A total of 27,028 bp were sequenced (fig. 1). The DNA sequences of the ancestral and evolved plasmids were identical. Thus, the reduction in kill zone size is due to changes in the host's chromosomal DNA and/or nonsequence based changes in the colicin plasmid.

Transcriptional Profiling

To attempt to identify candidate loci involved in the observed reduction in killing, global expression profiles of REL606 and CVE2 were compared. Isolates of each strain were obtained at transfers 0, 4, and 38 (0, ~27, and ~253 generations, respectively), and gene expression levels obtained in each of two replicate strains during exponential and stationary growth phases. A conservative measure of gene expression change was employed, which requires similar changes in each of two independently evolving cultures to occur. Under the selection criteria employed ($\alpha = 0.05$ and a fold change ≥ 1.5 , Guisbert et al. 2007), a total of 246 differentially expressed probe sets were identified.

CVE2 showed significantly different levels of expression at a total of 167 probe sets (17 by transfer 4 and an additional 150 by transfer 38; fig. 4). The expression levels measured during exponential and stationary phase growth revealed different patterns of change. A total of 125 probe

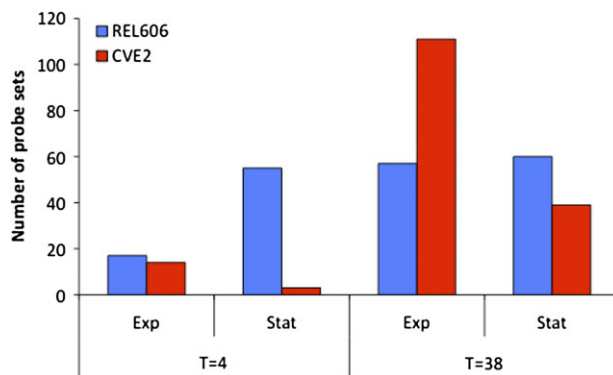


FIG. 4.—Number of loci differentially expressed in REL606 and CVE2 over time and in the stationary and exponential phase.

sets were identified in the exponential phase in CVE2 (14 by transfer 4 and 111 by transfer 38). In stationary phase, a total of 42 probe sets differed significantly (three by transfer 4 and 39 by transfer 38).

REL606 showed significantly different levels of expression at a total of 189 probe sets (72 by transfer 4 and an additional 117 by transfer 38) over the course of the experiment (fig. 4). During exponential growth, 74 probe sets were differentially expressed in REL606 with 17 expression differences appearing by transfer 4 and an additional 57 by transfer 38. In stationary phase, a total of 115 differentially expressed probe sets were identified in REL606 (55 by transfer 4 and an additional 60 by transfer 38).

The loci corresponding to the probe sets described above can be divided into 20 functional categories, representing COGs as defined by Tatusov et al. (2003) (www.ncbi.nlm.nih.gov/COG). Supplementary table 1 (Supplementary Material online) reports which loci fall into each COG. Supplementary table 1 (Supplementary Material online) also provides five novel categories created to represent functions involved specifically in colicin biology (Cascales et al. 2007) and two categories representing loci of unknown function.

Colicins target cells by recognizing specific cell surface receptors. To distinguish genes known to be involved in these functions, three categories were created that include genes involved in colicin E2 uptake. Category Z was created to include permease function, distinguishing sugar permeases (Zs) and other permeases (Zr). Many of the loci involved in colicin recognition are also involved in bacteriophage recognition (Cascales et al. 2007) and exposure to colicins affect phage-associated loci (Walker et al. 2004). To address the interactions between colicins and phage-associated genes, an additional category was created (Φ) that includes genes involved in the interaction between *E. coli* and its phage. Two other classes (Xa and Xb) were created that address the regulation and killing mechanism of colicin E2. Upon entry into the cell, colicin E2 kills the target cell by nonspecific DNA cleavage, which induces the SOS response regulated by the RecA/LexA response regulators (Walker et al. 2004). Finally, two classes were created for probe sets not classified (NC) and IRs.

Supplementary table 2 (Supplementary Material online) lists all loci that changed in CVE2 and, where relevant, in REL606. The most marked changes occurred by transfer 38 in the exponential phase (supplementary tables 1 and 2, Supplementary Material online) and include 17 phage-related genes, of which 12 were upregulated and five downregulated (supplementary table 2 [Supplementary Material online] and fig. 5A). In contrast, only one phage-related locus is found to change expression in REL606. Of the 12 upregulated phage-related loci in CVE2, eight have an assigned function (supplementary table 2, Supplementary Material online). Of these eight, at least five are loci that are expressed late in the phage lysogenic cycle and include the lysozyme of prophage DLP12 (*arrD*), an effector of mu-rein hydrolase (*yohJ*), a phage lambda B capsid protein (not named), a terminase (Locus tag c0953), and a putative regulator for late gene expression (c0978).

Loci involved in DNA repair (category Xa, fig. 5B) were also affected, although to a lesser degree, with one

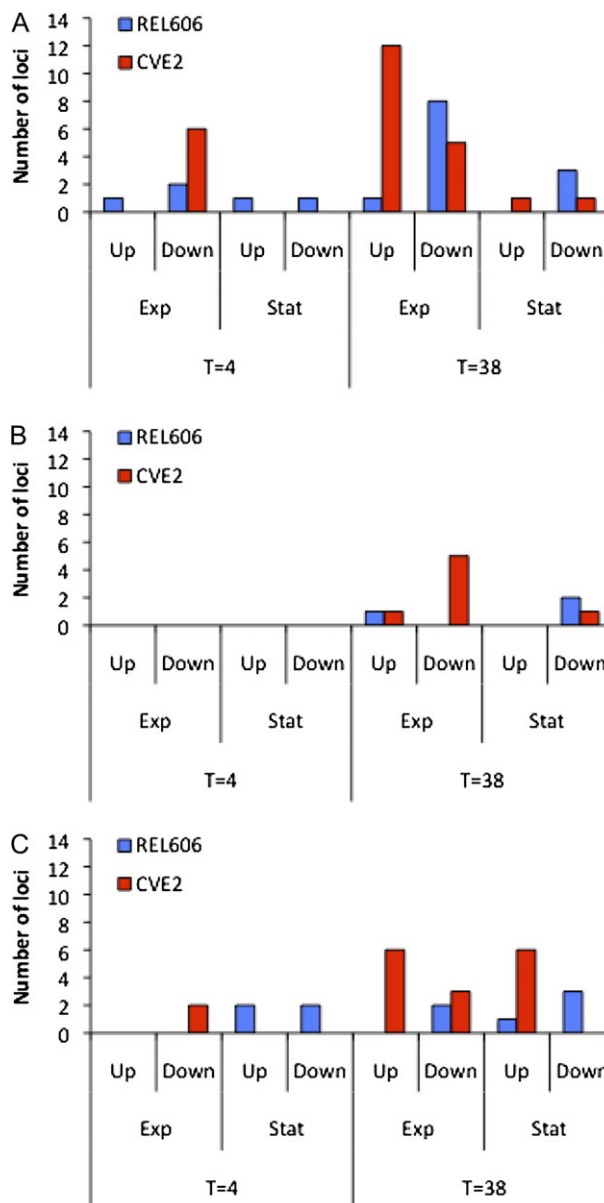


FIG. 5.—Number of phage- (A), DNA repair- (B), and nonsugar permeases- (C) loci differentially expressed in REL606 and CVE2 over time and in the stationary and exponential phase.

locus upregulated (*priA*) and five downregulated, including *sulA*, *dinI*, *umuD*, *recN*, and c0943, a *dinI*-like protein. Of the five downregulated loci, at least two, *sulA* and *umuD*, belong to the SOS regulon and are thus under LexA control. Nine loci encoding permeases (category Zr) were also affected, with six loci upregulated and three downregulated (fig. 5C). Within this group, the upregulated loci include the transporters *yabK* (thiamine ABC transport), *ompA* (an outer membrane protein), *ydeU* (an auto transporter), *yejO* (an auto transporter), *mchF* (microcin H47 transporter), and *mobD* (molybdenum transport protein). The downregulated loci include a lipoprotein of the OmpCFN family (*yddL*), a TonB-dependent protein (*yddB*), and a multidrug efflux system (*mdtD*). Furthermore, the three loci in category U (intracellular trafficking, secretion, and vesicular transport)

are *ydeU*, *yohK*, and *yejO*. In relation to carbohydrate metabolism (category G), three upregulated loci were found including one for a phosphotransferase system (PTS) system protein for maltose uptake (*malX*) and two involved in putative ribose uptake (*rbsA* and *rbsB*). The downregulated loci are involved in ribose uptake (*rbsD* and *rbsB*). Finally, genes involved in amino acid metabolism (category E) were affected. *YcjQ* (oxidoreductase) and *dsdA* (serine dehydratase) were upregulated and *yegB* (involved in multidrug efflux) was downregulated.

When CVE2 stationary phase was analyzed at transfer 38 (supplementary table 1, Supplementary Material online), six loci in category Zr (nonsugar permeases, fig. 5C) were upregulated, including *ydiZ* (involved in autoinducer transport), *fadL* (long-chain fatty acid transport), *mscL* (a mechanosensitive channel), *ecnB* (entericidin B, a bacteriolytic toxin), and *mgtA* involved in magnesium influx (supplementary table 2, Supplementary Material online). In category P (ion transport and metabolism), *bfr*, the iron storage protein bacterioferritin, is upregulated. As seen in the exponential growth phase, the loci involved in ribose metabolism (*rbsD* and *rbsP*) are downregulated. However, unlike the exponential phase maltose uptake is also downregulated (*malK*). In categories C and E, the genes involved in the *ast* operon are upregulated indicating increased arginine metabolism during the stationary phase in evolved CVE2.

At transfer 4, the main significant changes in gene expression in CVE2 were identified in exponentially growing cells (fig. 4). The loci affected are phage related (Φ , fig. 5A) and those that target DNA (category Xb), which were all downregulated. The phage-associated loci include a predicted murein hydrolase of prophage DLP12 (*rzpD*), rac prophage integrase (*intR*), a prophage tail fiber protein (c0977), a receptor for several phages and colicins (*tonA*), and two unknown proteins (z1781 and *yfiA*) encoded by prophage CP933N and CP933U (the translation inhibitor RaiA), respectively. The three downregulated loci in category Xb are the integrase from rac prophage (*intR*), a putative Tn5 transposase (z4324), and a restriction modification enzyme (z5948). Because colicinogeny is associated with protection against invading cells (Cascales et al. 2007), the restriction modification system is also classified in category V, a category specific for COGs involved in defense (supplementary table 2, Supplementary Material online).

Several of the loci with altered expression levels in CVE2 were also affected during the evolution of the control strain, REL606. These loci fall into two groups: those that respond in both strains in a similar way and those responding in opposite ways (supplementary table 2, Supplementary Material online). The COGs and loci responding differentially in CVE2 and REL606 include the following: in category P (ion uptake and metabolism) in the stationary phase at $T = 38$, *mgtA* was downregulated in REL606 and upregulated in CVE2. In category Q (secondary metabolite biosynthesis, transport, and catabolism), a predicted hydrolase (*ycaC*) was downregulated in the stationary phase in REL606 and upregulated in CVE2, albeit at different times. In category S (COGs with unknown function), *ybjX* was downregulated in the stationary phase in REL606 and upregulated in CVE2, again at different times. Additionally,

yddK, a predicted leucine-rich protein was upregulated in REL606 and downregulated in CVE2 in the exponential phase, and finally, a hypothetical protein (*smg*) was downregulated in the exponential phase at $T = 38$ in REL606 and upregulated in CVE2. In the category with NC loci, in the exponential phase, z0039 (a hypothetical protein) was upregulated in REL606 and downregulated in CVE2 at $T = 38$. In the stationary phase, *yogB* (a Mg^{2+} starvation inducible protein) was downregulated in REL606 at $T = 4$ and upregulated in CVE2 at $T = 38$, respectively. Finally, z5952, a hypothetical protein, was downregulated in REL606 at $T = 4$ and upregulated in CVE2 at $T = 38$. Very few significant changes in expression are identified between REL606 and CVE2 for genes involved in cell cycle control (D), nucleotide transport (F), secondary metabolites transport (Q), and signal transduction (T).

Discussion

The present study was designed to explore the phenotypic and genotypic changes that occur during the evolution of a colicinogenic strain in batch culture. To this end, an in vitro serial transfer experiment was conducted in which a colicinogenic strain (CVE2) and its plasmidless counterpart (REL606) were evolved for 253 generations. A significant decrease in killing ability was observed in CVE2 over time (fig. 2). This result is not surprising, given that colicin production is quite costly due to 1) the cost of replication and maintenance of the colicin plasmid (Feldgarden and Riley 1999 and this paper), 2) the cost of colicin production, and 3) the occurrence of autolysis of the producing cells (Braun et al. 1994).

When colicins are produced in an environment with no competitors, as is the case in this serial transfer experiment, it is reasonable to expect that the costs of colicin production will outweigh its benefits. A similar result was also obtained in an in vivo version of the present study, which also employed strains carrying colicin E2 plasmids, maintained in a mouse colon (Riley M and Gillor O, unpublished data). The colicin-producing strains evolved significantly reduced killing activity over 112 days. Prior to introduction of the colicin-producing strains in the mice, the native Gram-negative bacterial community was eliminated with antibiotic application. Thus, there was little or no competition with close relatives in both experimental designs, and in both cases killing levels significantly decreased over time.

According to Vasi et al. (1994), in serial transfer, V_{max} is a good reflection of the fitness of a population. Measures of V_{max} reveal a significant reduction in fitness of CVE2, relative to REL606 (fig. 3). Furthermore, the fact that these two strains differ only by the presence/absence of the colicin E2 plasmid suggests that the reduced fitness of CVE2 is due to possession of the colicin plasmid. The relative cost of colicinogeny is reflected by the reduced maximal growth rate (V_{max}) of CVE2 compared with REL606. The relative fitness of CVE2 compared with REL606 at the start of the experiment ($T = 0$) is $1.27/1.35 = 0.94$. This observed cost of colicin carriage has been observed in prior studies of colicin biology (Feldgarden and Riley 1999) and suggests that, in the absence of competition (which would

overwhelm the cost of plasmid carriage), there is a strong selective pressure acting to reduce the impact of the colicin plasmid's presence.

The observation that plasmid carriage can be costly is well known. For example, experiments by Bouma and Lenski (1988) show a reduction in fitness of strains carrying an antibiotic resistance plasmid. However, it is also well known that this cost can be ameliorated over time. For example, the same authors also evolved an *E. coli* host:plasmid combination for 500 generations and observed that evolution in the presence of a plasmid led to an increase in strain fitness. Similarly, using chemostats, Modi and Adams (1991) report a dramatic increase in growth rate of an *E. coli* strain carrying a plasmid over the course of 800 generations. In the present study, over the course of 38 transfers, REL606 and CVE2 experienced a similar relative increase in V_{max} (fig. 3). In CVE2, the observed increase in fitness and reduction in killing are not directly correlated, as the greatest changes in either measure do not occur at the same time points.

Several hypotheses can be invoked to explain the reduced killing phenotype observed in CVE2 over the course of this serial transfer experiment. One hypothesis posits that changes in the colicin gene cluster, or other plasmid-based changes, occur that alter the level of expression of the colicin gene or change its functional impact. For example, a reduction in the level of colicin protein produced would yield a smaller killing zone. Alternatively, simply reducing the level of expression of the lysis gene would result in less colicin protein reaching the target cells and thus a reduced killing zone in plate assays. Other possibilities include mutations affecting plasmid copy number or plasmid structure affecting expression of plasmid borne genes.

Whole-plasmid sequencing of three evolved pColE2 plasmids, one from each replicate culture, revealed no nucleotide polymorphisms compared with the ancestral plasmid. This assay involved determination of 6757 bp of sequence of which 2214 are specifically involved in colicin production, and 914 are involved in plasmid maintenance and copy number functions (fig. 1). With a neutral mutation rate of $\sim 1.44 \times 10^{-10}$ per bp per generation (Lenski et al. 2003), sequencing of three plasmids (20,271 bp), over 253 generations, and with a divergence time (in generations) of $-\log_2\{1 - (\text{substitutions}/20,271)\}/1.44 \times 10^{-10}$ (Elena et al. 2005), only 5.1×10^{-4} substitutions were expected. Thus, it is hardly surprising that no neutral mutations were observed. However, if a mutation occurred that resulted in a reduced kill zone, it would experience immediate and intense positive selection (due to the corresponding reduction in the cost of colicin production) and would have rapidly fixed in a population of the size employed in this study. Therefore, it is reasonable to assume that evolution resulting in reduced killing must have occurred on the host chromosome. Indeed, Bouma and Lenski (1988) showed that a host:plasmid association underwent significant adaptation during 500 generations of evolution and that this adaptation was the result of evolution of the host chromosome, not the plasmid.

To identify evolutionary responses in chromosomally encoded functions, a transcriptome analysis was undertaken. The transcriptome of CVE2 at transfers 4 and 38 were compared with that of the ancestral strain (CVE2 at

$T = 0$). Changes responsible for a reduction in kill zone will be absent in the REL606 evolved control strain. The experimental and control strains shared some gene expression changes, which are likely loci responding to adaptation to the culture conditions and media. These loci are mainly involved in category G (carbohydrate transport and metabolism), and, for ribose uptake, in category Zs (sugar permeases). In all cultures analyzed, a downregulation of the *rbs* operon and its regulator (category K) took place. This culture-based effect has been observed previously (Cooper et al. 2003; Pelosi et al. 2006). In fact, over longer evolutionary periods, this operon is lost all together in REL606 (Cooper et al. 2001). Additionally, in all cultures, loci involved in maltose uptake were upregulated in the exponential phase. This increase in expression of *malX* (maltose and glucose-specific IIBC component of PTS system) suggests an increase in maltose and glucose metabolism. Furthermore, in all evolving cultures, *bfr*, a locus involved in iron uptake, was upregulated in the stationary phase. In the category of the NC loci, two loci, *ymdA* and *c1602* (predicted proteins) were downregulated in REL606 and CVE2, in the exponential phase.

Loci that specifically changed during the evolution of CVE2 include phage-related loci (Φ) and Mg^{2+} (categories P and Zr) metabolism (supplementary tables 1 and 2 [Supplementary Material online], fig. 5A and C). Responses to colicins often involve ion uptake (Cascales et al. 2007). In this study, we found that ion transporters were affected during evolution in the presence of pColE2. A surprising finding is the change in Mg^{2+} uptake that occurred in both REL606 and CVE2. In REL606, the *mgtA* locus is downregulated in the exponential and stationary phase, at $T = 4$ and $T = 38$, indicating a sufficient amount of available Mg^{2+} . In CVE2, however, the same locus is upregulated in stationary phase at $T = 38$.

The observation of the upregulation of *psiF* and *mgtB* (loci responsive to Mg^{2+} starvation) in CVE2 emphasizes the increased need for Mg^{2+} during CVE2 evolution. One explanation for this increased need for Mg^{2+} may be found in the biology of E2 colicins. Colicins belong to the group of homing nucleases with an HNH motif (Sui et al. 2002; Hsia et al. 2004; Stoddard 2005). These motifs resemble distorted zinc-finger motifs with a single metal ion between them. The interesting difference with DNase colicins is that they require high concentrations of Mg^{2+} or Ca^{2+} rather than Zn^{2+} . For example, Mg^{2+} has been identified as responsible for colicin E9 activity (Walker et al. 2002). Additionally, activity of E7 is enhanced in the presence of Mg^{2+} (Sui et al. 2002). The ability to bind Mg^{2+} may interfere with normal cellular functions. The decrease in killing ability may be a result of competition of the host and the colicin for available Mg^{2+} . Under the test conditions employed, CVE2 apparently faces a shortage of Mg^{2+} , leading to changes resulting in an upregulation of Mg^{2+} starvation genes and a downregulation of homing endonuclease activity with HNH motifs.

Expression of 25 phage-associated loci (category Φ , fig. 5A) is affected in the evolving CVE2 populations. These loci have no overlap with affected phage-related loci in REL606 indicating different phage-based evolutionary responses in the two lineages. That the biology of phages and colicins has much in common has been known for some

time and is not restricted to *E. coli*. For example, R pyocins and cepaciocins recognized in *Pseudomonas aeruginosa* and *Burkholderia cepacia* resemble phage tail-like proteins (Govan and Harris 1985; Michel-Briand and Baysse 2002). Furthermore, a genetic association of bacteriocin and phage genes in *Klebsiella* has been revealed (Chavan et al. 2005), indicating the involvement of phage in the diversification of bacteriocins. The findings of Walker et al. (2004) also support a closer association between phage and bacteriocin biology, albeit on the physiological level. These authors found that exposure of *E. coli* K12 to colicin E3 leads to increased expression in phage-associated and other auxiliary loci.

In CVE2, most phage-associated loci are affected during the exponential growth phase by transfer 38, and most of these loci are upregulated. Of the eight loci with an assigned function, at least five (two of which are holins or effectors of holins, one terminase involved in packaging DNA, one capsid protein, and one regulator of late gene expression) appear to be involved in late gene expression of temperate phages (Casjens 2003). An increase in late phage gene expression is surprising because REL606 is not known to contain active phages. This may indicate that during the evolution of CVE2, conditions are met that lead to the activation of previously unknown inducible prophages.

The observed increase in V_{max} and expression of late phage genes suggest an increased probability of excision of a cryptic prophage during evolution of a colicinogenic strain. Conversely, downregulation of holin expression and a putative tail fiber gene at $T = 4$ are correlated with a decrease in V_{max} at this transfer point (fig. 3 and supplementary table 2, Supplementary Material online). Interestingly, Bossi et al. (2003) showed that a deletion mutant for the “int-xis” region (which prevents prophage excision) in phage Gifsy-2 of *Salmonella* was no longer able to outgrow the reference (wild-type) strain in a mixed culture. This may indicate that the extrachromosomal form of the phage is needed for an increase in competitiveness.

It was anticipated that loci involved in DNA damage repair (Categories L and Xa) might be the target of selection during prolonged growth in the presence of colicin toxin, particularly because exposure of a sensitive culture to colicin E9, a DNase, induces the SOS response (Walker et al. 2004). Furthermore, weak constitutive expression of colicin may result in increased levels of DNA damage (Cascales et al. 2007). Thus, it was predicted that the SOS response would be induced over the course of this experiment. However, neither significant differences in *recA* and *lexA* expression was seen nor was there an increase in expression of DNA repair genes. In fact, these genes experienced a reduction in gene expression. Most colicin-encoding genes contain LexA binding sites in their promoter (Gillor et al. 2008); thus, the observed lack of a SOS response may explain the reduction in killing because colicin genes are under direct control of the SOS regulon, and LexA represses expression of these loci. An increased affinity of LexA to the LexA box would not only lead to a downregulation of the SOS inducible DNA repair genes but also to an increased repression of colicin gene expression. Subsequently, many DNA repair genes (category Xa, fig. 5B), including *umuD*, *priA* and *rmuA*, *dinI*, *recN*, and *sulA*, were downregulated in the evolved CVE2 strain.

This study explores the population dynamics of a colicin-producing strain of *E. coli* exposed to serial transfer conditions, with no competitors, over 253 generations. As was previously observed in a similar experiment carried out in vitro, the strain evolved a reduction in killing activity and a corresponding increase in fitness, relative to the ancestral strain. We speculate that the lack of competitive interactions results in strong selection for a decrease or elimination of colicin production. No changes were observed in the DNA sequence of the evolved colicin plasmids. However, a set of chromosomally encoded loci experienced changes in gene expression that were positively correlated with the reduction in killing. Further studies are required to more accurately identify the precise molecular changes that result in the observed reduction in killing. Perhaps these future studies will also reveal why selection results in changes in gene expression, rather than simple elimination of the plasmid itself. Although colicins are touted as one of the most intensively studied of bacterial weapons, it is clear that there is much we still don't know about how this exquisite system of biodefense evolved.

Supplementary Material

Supplementary tables 1 and 2 are available at *Genome Biology and Evolution* online (http://www.oxfordjournals.org/our_journals/gbe/).

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