Adaptation to the fitness costs of antibiotic resistance in *Escherichia coli*

STEPHANIE J. SCHRAG*, VÉRONIQUE PERROT AND BRUCE R. LEVIN

Department of Biology, Emory University, Atlanta, GA 30322, USA

SUMMARY

Policies aimed at alleviating the growing problem of drug-resistant pathogens by restricting antimicrobial usage implicitly assume that resistance reduces the Darwinian fitness of pathogens in the absence of drugs. While fitness costs have been demonstrated for bacteria and viruses resistant to some chemotherapeutic agents, these costs are anticipated to decline during subsequent evolution. This has recently been observed in pathogens as diverse as HIV and *Escherichia coli*. Here we present evidence that these genetic adaptations to the costs of resistance can virtually preclude resistant lineages from reverting to sensitivity. We show that second site mutations which compensate for the substantial (14 and 18% per generation) fitness costs of streptomycin resistant (*rpsL*) mutations in *E. coli* create a genetic background in which streptomycin sensitive, *rpsL*$^+$ alleles have a 4–30% per generation selective disadvantage relative to adapted, resistant strains. We also present evidence that similar compensatory mutations have been fixed in long-term streptomycin-resistant laboratory strains of *E. coli* and may account for the persistence of *rpsL* streptomycin resistance in populations maintained for more than 10 000 generations in the absence of the antibiotic. We discuss the public health implications of these and other experimental results that question whether the more prudent use of antimicrobial chemotherapy will lead to declines in the incidence of drug-resistant pathogenic microbes.

1. INTRODUCTION

Recent laboratory studies of microbes as diverse as HIV (Borman *et al*. 1996) and *E. coli* (Bouma & Lenski 1988; Lenski *et al*. 1994; Schrag & Perrot 1996) challenge a widespread view (Levy 1994; OTA 1995; WHO 1995) that the frequency of drug-resistant pathogens will decline if drug usage is reduced. This view implicitly assumes that resistant strains have a competitive disadvantage relative to sensitive strains in the absence of drugs. The above-cited studies demonstrate that even when pathogen resistance is associated with substantial fitness costs, subsequent pathogen evolution reduces these burdens without lowering the level of resistance. In at least one case, adaptations to the cost of an antibiotic-resistance encoding plasmid in *E. coli* resulted in resistant bacteria which were more fit than the plasmid-free parental strain even in the absence of the antibiotic (Bouma & Lenski 1988). Furthermore, in at least two studies (Borman *et al*. 1996; Schrag & Perrot 1996), this rapid adaptation to the cost of resistance occurred in the absence of drugs selecting for resistance.

Here, we present evidence that adaptations to costs of resistance, in addition to reducing the fitness costs of resistance, can create a genetic background that confers a selective disadvantage on sensitive alleles. Using *rpsL* strains of *E. coli*, we demonstrate that second site mutations which compensate for the fitness costs associated with resistance create a genetic background in which wild-type, streptomcyin-sensitive alleles (*rpsL*$^+$) have a marked selective disadvantage. Stated another way, these compensatory mutations establish an 'adaptive valley' (Wright 1932; Dobzhansky 1951) which virtually precludes the evolved, resistant lineages from reverting to drug sensitivity. We present retrospective evidence which suggests that compensatory evolution and a resulting 'adaptive valley' may have contributed to the stability of streptomycin resistance in long-term, antibiotic-free laboratory cultures of *E. coli* B. We conclude with a brief discussion of the clinical and public health implications of these and related experimental results.

2. METHODS

(*a*) *Strains*

Our wild-type, streptomycin-sensitive strain was *E. coli* CAB281, an *E. coli* K1/K12 chimera (χM1002 in Bloch & Rode (1996)). Spontaneous streptomycin-resistant mutants of this strain, STR1 and STR2, differed from the wild-type by single base substitutions at codon 42 of the *rpsL* gene resulting in threonine and aspargine, respectively, instead of the wild-type lysine. STR11 and STR12 were clones of the parental strain STR1, which were maintained independently in serial transfer culture in the absence of streptomycin for 180 generations (as described in Schrag & Perrot (1996)). Similarly,

*Present address: Respiratory and Enteric Viruses Branch, DVRD, Mail Stop C-22, Centers for Disease Control, 1600 Clifton Road, Atlanta, GA 30333, USA (zha6@cdc.gov).

STR21 and STR22 were 180-generation evolved strains derived independently from STR2 . The *rpsL* sequence in all four evolved strains did not differ from the parental sequence (Schrag & Perrot 1996). Our strain of *E. coli* B was a subclone of strain Bc251 ($T6^r$, Str^r) (Lederberg 1966) donated by S. Lederberg. REL4548 (Lenski & Travisano 1994), a 10 000-generation evolved descendant of this *E. coli* B strain, was donated by R. Lenski.

(*b*) *P1 phage-mediated transduction*

P1 transduction was performed using P1*vir*, donated by D. Thaler. Phage were grown on an Str^s donor strain, *E. coli* K12 CAG18556 (Singer *et al*. 1989) provided by C. Gross, which had a kanamycin-resistance marker (Kan^r) 1 min from *rpsL*. Lysate preparation and P1 transduction followed Miller (Miller 1972). Kan^r colonies resulting from transduction experiments were streaked for single colonies on LB plates supplemented with sodium citrate and kanamycin. Resulting single colonies were screened for high-level streptomycin resistance ($>400\,\mu g\,ml^{-1}$). Kan^r Str^s candidates and at least two randomly chosen Kan^r Str^r candidates were further screened for strain-identifying markers, and stored in 15% glycerol at -80° C.

(*c*) *Estimating bacterial fitness*

The fitness of a Kan^r Str^s transductant relative to a Kan^r Str^r transductant of the same recipient genetic background was estimated by measuring changes in the frequency of the streptomycin-sensitive transductants in direct competition with a streptomycin-resistant transductant. Each competition was initiated with equal frequencies of Kan^rStr^s and Kan^rStr^r transductants in 50 ml flasks containing 10 ml of culture medium. Competitions were maintained for at least 33 generations of growth, by transferring daily 0.025 ml of stationary phase culture to fresh glucose-limited ($500\,\mu g\,ml^{-1}$) Davis minimal medium (Carlton & Brown 1981) supplemented with $MgSO_4$ and thiamine in excess and nicotinic acid to a final concentration of $10\,\mu g\,ml^{-1}$. In cases where the streptomycin-sensitive transductants had a strong selective disadvantage, the initial ratio of sensitive to resistant strains was 100:1, rather than 1:1, to increase the number of generations over which changes in the relative frequency of the Str^s strain could be measured. The selection coefficient of a given strain was then estimated by the regression coefficient of the natural log of the ratio of sensitive to resistant strains plotted against time (generations of competition) (Lenski 1991). Relative fitness per bacterial generation was calculated as one minus the selection coefficient (Lenski 1991).

For each Str^s transductant, a single pairwise competition was performed against each of two independent Str^r transductants of the same recipient. Because the fitness of independent Kan^rStr^r transductants relative to their unmanipulated Str^r recipient was not found to vary significantly (unpublished results), a single regression was fitted through the points from both pairwise competitions instead of calculating a regression coefficient for each separately. Reported standard errors and p values are calculated for the regression coefficient.

3. RESULTS

Using P1-mediated transduction, we replaced the *rpsL* alleles in a collection of Str^r strains of *E. coli* CAB281 with the wild-type, sensitive allele. Two strains from this collection (STR1 and STR2) were newly isolated Str^r mutants with 14 and 19% per generation fitness disadvantages relative to the wild-type strain (Schrag & Perrot 1996). The remaining strains, evolved descendants of these two mutants, carried second-site mutations which compensated for these fitness disadvantages without reducing the resistance to streptomycin (Schrag & Perrot 1996). The wild-type *rpsL* allele introduced into these strains came from a Str^s *E. coli* K12 donor strain carrying a Kan^r marker 1 min from the *rpsL* gene.

In the unevolved recipient strains, STR1 and STR2, we obtained a modest frequency of Kan^r Str^s co-transductants, as would be expected from the 1 min map distance between these two genes (table 1). In one of the evolved strains, STR21, we obtained a similar frequency of co-transductants. In the evolved strains STR12 and STR22, however, the rate of co-transduction of the Kan^r and Str^s alleles was substantially lower, and in the last of these evolved strains (STR11), we failed to obtain any Kan^r Str^s co-transductants after screening nearly 4000 Kan^r colonies. This suggests that the Str^s allele in evolved genetic backgrounds reduced bacteria viability.

To test directly whether a fitness cost was associated with wild-type, $rpsL^+$ alleles in strains bearing mutations compensating for the costs of resistance, we measured the fitness of wild-type alleles relative to resistant alleles in

Table 1. *Number of Kan^r Str^s co-transductants obtained using unevolved and evolved streptomycin-resistant* E. coli *CAB281 strains and an* E. coli *B strain as recipients: P1-mediated transduction was performed as described in the methods, using a Str^s donor strain with a Kan^r-marker* 1 min *from the* rpsL *gene*

recipient strain[a]	evolutionary status	number of Kan^r transductants screened	number of Kan^rStr^s co-transductants	frequency of Kan^rStr^s co-transductants
STR1	unevolved	555	13	0.02
STR2	unevolved	58	3	0.05
STR11	evolved	3968	0	0.0
STR12	evolved	308	1	0.003
STR21	evolved	204	3	0.015
STR22	evolved	1185	3	0.003
E. coli B/6	laboratory strain with a 25-year history	290	7	0.024

[a]All strains are the same as those described by Schrag & Perrot (1996), except the *E. coli* B strain which is described by S. Lederberg (1966) and has been maintained in this laboratory since 1970.

both unevolved and evolved genetic backgrounds by competing Kan^r Str^s transductants against randomly chosen Kan^r Str^r transductants obtained from the same genetic background.

As anticipated from our original observations (Schrag & Perrot 1996), the Str^s allele had a marked advantage over Str^r alleles in the unevolved genetic background (figure 1*a*). Moreover, the fitness costs associated with these resistance alleles in transductants were similar to the 14% and 19% per generation fitness costs estimated in previous competition experiments between wild-type and resistant mutant strains (Schrag & Perrot 1996).

In contrast to this strong advantage of the Str^s allele in the unevolved genetic background, in evolved strains carrying second-site compensatory mutations the Str^s allele had a significant fitness disadvantage relative to the resistant alleles (figure 1*b*). In the evolved genetic background of STR21, the fitness disadvantage of the Str^s allele was modest, 3% per generation. In the background of the other two evolved strains, STR12 and STR22, the fitness disadvantage of Str^s was much greater, ranging between 20 and 25% per generation. The difference between the disadvantages of the Str^s allele in two backgrounds (STR21 and STR22) evolved from the same parental strain (STR2) is consistent with previous evidence (Schrag & Perrot 1996) suggesting that more than one second site mutation can compensate for the fitness costs of a given Str^r, *rpsL* allele.

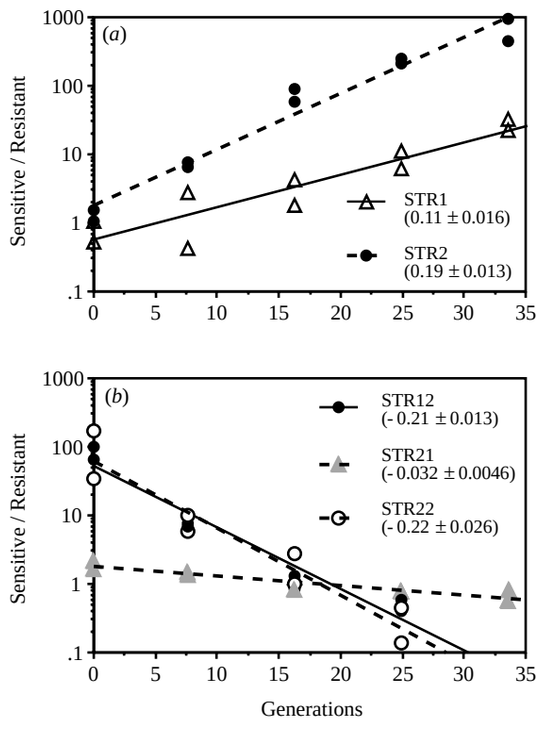

Figure 1. Change in the relative frequency of Kan^r Str^s transductants relative to Kan^r Str^r transductants of the same recipient strain in pairwise competition experiments (see §2). The indicated strain names refer to the genetic background of the recipients. (*a*) The effect of the Str^s allele in the unevolved genetic background. (*b*) The effect of the Str^s allele in the evolved genetic background. Estimates of selection coefficients, shown in parentheses below the strain names, are based on at least two pairwise competitions per strain. These estimates were calculated by linear regression (shown by lines on the graphs) as described in §2. Standard errors were based on the regression and all p values of the regressions were less than 0.00015.

If the present observations are general, then *rpsL*$^+$ alleles should be associated with a fitness cost in laboratory *rpsL* strains that have been maintained for extensive periods, even if these strains have been maintained in the complete absence of streptomycin. We tested this prediction using a laboratory strain of *E. coli* B (Lederberg 1966) which was resistant to streptomycin when first received in our laboratory in 1970, and which has been used most recently by R. Lenski and colleagues to initiate a series of long-term experimental evolutionary studies (Lenski *et al*. 1991; Lenski & Travisano 1994; Travisano *et al*. 1995; Elena *et al*. 1996; Travisano & Lenski 1996). In these experiments, this strain was allowed to adapt over the course of 10 000 generations to serial transfer culture in streptomycin-free, glucose-limited medium. Despite significant changes in cell size, fitness, and physiology over this period (Lenski *et al*. 1991; Lenski & Travisano 1994), we found using standard DNA amplification and sequencing methods (described in Schrag & Perrot (1996)) that the *rpsL* sequences of the 10 000 generation strain and its parental *E. coli* B strain are identical. In place of the wild-type lysine at codon 42, both strains carry a point mutation resulting in ACA that codes for threonine.

Furthermore, when we replaced this *rpsL* allele in the unevolved, parental strain by the wild-type, Str^s allele, five Kan^r Str^s transductants had an average fitness cost of 26% (range 20.3–30%) per generation relative to Kan^r Str^r transductants. This provides evidence that mutations compensating for the fitness costs of streptomycin resistance arose in this strain prior to the initiation of long-term evolution studies by Lenski *et al*. However, unlike our controlled experiments with *E. coli* CAB281, in this retrospective investigation we do not have the immediate Str^s ancestor of this *E. coli* B Str^r lineage, and therefore cannot directly demonstrate compensatory mutations in this strain.

4. DISCUSSION

Mutations which decreased the fitness costs of chromosomal streptomycin-resistance in *E. coli* also created a genetic background in which streptomycin-sensitive revertants have a strong selective disadvantage. In the context of the Wrightian adaptive landscape metaphor (Wright 1932; Dobzhansky 1951), the genetic changes that compensate for the cost of resistance establish a valley that separates the adaptive peaks of drug sensitivity and fitness-compensated resistance (figure 2). Not only are at least two mutations required for an evolved resistant strain to become a high-fitness sensitive strain, individually each of these mutations reduces the fitness of that strain. As a result, it would be very difficult for an evolved resistant lineage to become sensitive, even in the absence of the drug.

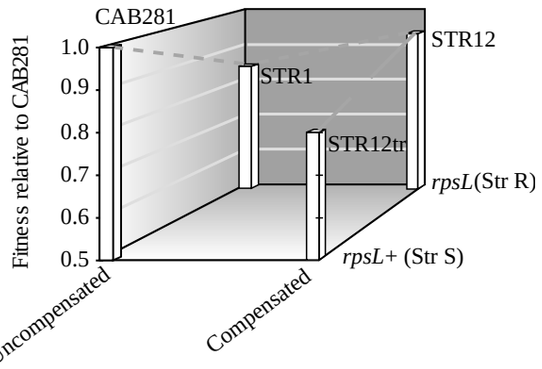

Figure 2. Schematic representation of the adaptive landscape determined by the four classes of strains studied, based on data obtained for STR12. Once a compensatory mutation is fixed in a resistant strain, resistant-compensated and wild-type (sensitive-uncompensated) strains occupy different adaptive peaks separated by a valley. The fitness of STR12tr, which is an Str^s transductant of STR12, relative to CAB281 was estimated by direct competition as described in the methods. Because the Kan^r marker STR12tr carries may have influenced the fitness of this strain relative to Kan^s CAB281, we estimated the fitness cost of the Kan marker in the STR12 background with the following results: fitness of STR12 relative to CAB281: 0.77 ± 0.02; fitness decrease due to the Kan^r marker: 0.02 ± 0.004. We therefore estimated the relative fitness of STR12tr as 0.79 relative to CAB281.

From a clinical and public health perspective, these results point to another potentially general reason why reductions in antimicrobial usage may not lead to rapid declines in the incidence of resistant pathogens. Compensation for the fitness costs of resistance has been shown to occur both in the presence and in the absence of drug selection (Bouma & Lenski 1988; Lenski *et al*. 1994; Borman *et al*. 1996; Schrag & Perrot 1996). In the latter case, mathematical models suggest that compensatory evolution, rather than reversion to sensitivity, is expected to occur as long as the rate of compensatory mutations exceeds that of reversion to sensitivity, and only a fraction of the population is transferred to colonize a new host (Levin *et al*. 1997).

Our observations that such compensation confers a selective disadvantage on sensitive revertants are specific to chromosomal streptomycin resistance in *E. coli*. Furthermore, this may be a specific feature of ribosomal resistance mutations due to the complexity of ribosomal protein interactions, and further studies of other target mutations observed in clinical isolates (e.g. alterations of RNA polymerase, or gyrase) are required to establish the generality of our observations. Nonetheless, evidence from other studies suggests that analogous adaptive valleys between sensitive and adapted, resistant peaks may exist for other cases of resistance. In the case of plasmid-borne resistance (Bouma & Lenski 1988; Lenski *et al*. 1994), a tetracycline resistance-encoding plasmid which initially reduced the fitness of host bacteria in the absence of tetracycline, actually augmented bacterial fitness after 500 generations of culture in association with the bacterial host. Adaptive valleys of the sort observed here are also likely to be present in cases where resistance to clinical levels of chemotherapeutic agents is achieved through the sequential substitution of multiple mutations, each of which is favoured in the presence of the drug (e.g. AZT resistance (Larder & Kemp 1989; Kellam *et al*. 1994); protease inhibitor resistance (Borman *et al*. 1996)). In these cases, reversion to sensitivity requires the substitution of multiple mutations. It is likely, and there is suggestive evidence in a study of HIV resistance to a protease inhibitor (Borman *et al*. 1996), that reversions at these loci will not be selected because they do not improve, and often reduce pathogen fitness.

While this evidence comes from *in vitro* systems, epidemiological studies in the case of HIV also suggest that resistant strains will not wane rapidly with reduced drug use. An AZT-resistant HIV strain transmitted by heterosexual contact has persisted as the dominant virus population in the recipient host who was never treated with AZT, showing that AZT-resistant strains persist for longer than anticipated in the absence of drug treatment (Conlon *et al*. 1994). Furthermore, AZT-resistant strains have been slow to wane in patients who have discontinued AZT-treatment (Boucher *et al*. 1993).

Despite this, we do not believe these observations should be construed as arguments for the futility of controlling resistance by reduced and more prudent drug use. While the mechanisms discussed here will substantially retard the rate at which any given lineage of resistant pathogens declines even in the complete absence of drug usage, they need not preclude a return to drug sensitivity. Population models have shown that as long as populations of pathogenic microbes are polymorphic and include sensitive as well as resistant lineages, and as long as these sensitive strains have some competitive advantage over resistant strains, reductions in drug usage will increase the frequency of the sensitive strains and the likelihood of these lineages replacing resistant lineages (Levin *et al*. 1997). Furthermore, the rate and extent to which pathogens adapt, by compensatory mutations and other mechanisms, to an environment where drugs are prevalent is expected to decrease with decreased drug usage. Increasing the number of *in vivo* and epidemiological studies, where spatial refuges and migration of drug-sensitive pathogens in particular might play important roles, will be particularly important in understanding how to control drug resistance within pathogen populations.

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REFERENCES

Bloch, C. A. & Rode, C. K. 1996 Pathogenicity island evaluation in *Escherichia coli* K1 by crossing with laboratory strain K-12. *Infect. Immunity* **64**, 3218–3223.

Borman, A. M., Paulous, S. & Clavel, F. 1996 Resistance of human immunodeficiency virus type 1 to protease inhibitors: selection of resistance mutations in the presence and the absence of the drug. *J. Gen. Virol*. **77**, 419–426.

Boucher, C. A. B., Van Leeuwen, R., Kellam, P., Schipper, P., Tijnagel, J., Lange, J. M. A. & Larder, B. A. 1993 Effects of discontinuation of zidovudine treatment on zidovudine sensitivity of Human Immunodeficiency Virus Type 1 isolates. *Antimicrob. Agents Chemother.* **37**, 1525–1530.

Bouma, J. E. & Lenski, R. E. 1988 Evolution of a bacteria–plasmid association. *Nature* **335**, 351–352.

Carlton, B. C. & Brown, B. J. 1981 Gene mutation. In *Manual of methods for general microbiology* (ed. Gerhardt), pp. 222–242. Washington, DC: American Society for Microbiology.

Conlon, C. P., Klenerman, P., Edwards, A., Larder, B. A. & Phillips, R. E. 1994 Heterosexual transmission of Human Immunodeficiency Virus Type 1 variants associated with zidovudine resistance. *J. Infect. Dis.* **169**, 411–415.

Dobzhansky, T. 1951 *Genetics and the origin of the species*. New York: Columbia University Press.

Elena, S. F., Cooper, V. S. & Lenski, R. E. 1996 Punctuated evolution caused by the selection of rare beneficial mutations. *Science* **272**, 1802–1804.

Kellam, P., Boucher, C. A. B., Tijnagel, J. M. G. H. & Larder, B. A. 1994 Zidovudine treatment results in the selection of human immunodeficiency virus type 1 variants whose genotypes confer increasing levels of resistance. *J. Gen. Virol.* **75**, 341–351.

Larder, B. A. & Kemp, S. D. 1989 Multiple mutations in HIV-1 reverse transcriptase confer high-level resistance to zidovudine (AZT). *Science* **246**, 1155–1158.

Lederberg, S. 1966 Genetics of host-controlled restriction and modification of deoxyribionucleic acid in *Escherichia coli*. *J. Bact.* **91**, 1029–1036.

Lenski, R. E. 1991 Quantifying fitness and gene stability in microorganisms. In *Asseessing ecological risks of biotechnology.* (ed. L. R. Ginzburg), pp. 173–192. Boston: Butterworth-Heinemann.

Lenski, R. E., Rose, M. R., Simpson, S. C. & Tadler, S. C. 1991 Long-term experimental evolution in *Escherichia coli*. I. Adaptation and divergence during 2000 generations. *Am. Nat.* **183**, 1315–1341.

Lenski, R. E., Simpson, S. C. & Nguyen, T. T. 1994 Genetic analysis of a plasmid-encoded, host genotype-specific enhancement of bacterial fitness. *J. Bact.* **176**, 3140–3147.

Lenski, R. E. & Travisano, M. 1994 Dynamics of adaptation and diversification: a 10 000-generation experiment with bacterial populations. *Proc. Natn. Acad. Sci. USA* **91**, 6808–6814.

Levin, B. R., Lipsitch, M., Perrot, V., Schrag, S., Antia, R., Simonsen, L., Moore, N. & Stewart, F. M. 1997 The population genetics of antibiotic resistance. *Clinical Infectious Disease* **24** (Suppl. 1), S9–S16.

Levy, S. B. 1994 Balancing the drug-resistance equation. *Trends Microbiol.* **2**, 341–343.

Miller, J. H. 1972 *Experiments in molecular genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

OTA 1995. *Impacts of antibiotic-resistant bacteria*. Washington, DC: Office of Technology Assessment, United States Congress.

Schrag, S. J. & Perrot, V. 1996 Reducing antibiotic resistance. *Nature* **381**, 120–121.

Singer, M., Baker, T. A., Schnitzler, G., Deischel, S. M., Goel, M., Dove, W., Jaacks, K. J., Grossman, A. D., Erickson, J. W. & Gross, C. A. 1989 A collection of strains containing genetically linked alternating antibiotic resistance elements for genetic mapping of *Escherichia coli*. *Microbiol. Rev.* **53**, 1–24.

Travisano, M. & Lenski, R. E. 1996 Long term experimental evolution in *Escherichia coli*: IV. Targets of selection and the specificity of adaptation. *Genetics* **143**, 15–26.

Travisano, M., Mongold, J. A., Bennett, A. F. & Lenski, R. E. 1995 Experimental tests of the roles of adaptation, chance, and history in evolution. *Science* **267**, 87–90.

WHO 1995. *WHO scientific working group on monitoring and management of bacterial resistance of anitmicrobial agents*. Geneva: World Health Organization.

Wright, S. 1932 The roles of mutation, inbreeding, crossbreeding, and selection in evolution. *Proc. Sixth Int. Congress Genetics* **1**, 356–366.

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