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# Spontaneous and on point: Do spontaneous mutations used for laboratory experiments cause pleiotropic effects that might confound bacterial infection and evolution assays?

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**One sentence summary:** This review examines the pleiotropic effects of point mutations conferring antibiotic resistance, and the implications of using these resistance mutants in both *in vitro* and *in vivo* assays.

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## ABSTRACT

Many selectable phenotypes in microbial systems, including antibiotic resistance, can be conferred by single point mutations. This is frequently exploited in research, where the selection and use of microbial mutants that are spontaneously resistant to antibiotics like rifampicin and streptomycin facilitate the recovery and/or quantification of a target microbe. Such mutations are commonly employed as genetic markers for *in vitro* and *in vivo* experiments, often with little consideration as to the ultimate system-level impact of these single nucleotide mutations on the physiology of the microbe. There is substantial literature on the pleiotropic effects of point mutations conferring antibiotic resistance; yet, it is unclear whether this work is considered by the research communities outside of the discipline. This review examines some of the known pleiotropic effects of point mutations that provide selectable resistance markers, and how these mutations may impact general physiology and growth in host and non-host environments.

**Keywords:** antibiotic resistance; point mutation; spontaneous; pleiotropic effects; rifampicin; streptomycin

## INTRODUCTION

There is a long history in microbiological research of isolating spontaneous mutants conferring selectable phenotypes for use in experimental assays. Assays evaluating host–microbe interactions or microbial physiology and fitness are greatly facilitated by the use of selectable markers, since they enable the researcher to isolate and recover the specific microbe of interest. The most widely used selectable markers in microbiology are those conferring antibiotic resistance. The point mutations that lead to these selectable phenotypes usually confer antibiotic resistance by altering the structure of the drug target, thereby preventing or reducing the ability of the antibiotic to interfere with its function (Lambert 2005). Spontaneous mutants carrying these mutations are isolated by high-density plating of the wild-type isolate on the antibiotic of interest, and are often used as analogues to the wild-type isolate under the experimental conditions being evaluated. Because the mutations that confer these phenotypes frequently occur within essential genes whose products have central and often global roles in housekeeping functions, and these mutations do not

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eliminate these essential processes, the global impact on cellular processes and regulation may not always be considered (Lambert 2005). For example, streptomycin binds the S12 protein of the 30S subunit, interfering with translation mechanisms required for protein elongation and altering accuracy (Sreevatsan *et al.* 1996). Mutations conferring streptomycin resistance result from point mutations that alter amino acid 43 within the *rpsL* gene, or from point mutations that introduce conformational changes to a pseudoknot structure in the 16S rRNA that is linked to ribosomal protein S12 (Finken *et al.* 1993; Springer *et al.* 2001; Olkkola *et al.* 2010; Poggi, Oliveira de Giuseppe and Picardeau 2010). These mutations do not strictly compromise cellular viability and are not lethal, yet the impact on translation efficiency at the systems level may not be considered. Similarly, the target of the antibiotic rifampicin is the beta subunit encoded by the RNA polymerase gene, *rpoB,* with high-level resistance being conferred by S522L, H526N and S574L (Jin and Gross 1988; Aubry-Damon *et al.* 2002), while resistance to the quinolones, which target DNA gyrase, is acquired through multiple point mutations in *gyrA* and *gyrB* genes (Yoshida *et al.* 1990). Because in all these cases the resistance-conferring mutations do not compromise base functionality of critical housekeeping proteins, the resulting mutants are often used as wild-type proxies in experimental assays; however, is this a good strategy?

The use of spontaneously resistant antibiotic mutants has been expediting the recovery of microbes and facilitating the quantification of microbial growth in a variety of experimental assays across many microbiological disciplines; however, much research has shown the pleiotropic, system-level effects of point mutations can alter general physiology in mutants. This review will examine the pleiotropic effects observed in spontaneous antibiotic-resistant mutants, including how point mutations can affect key regulatory mechanisms that may critically impact experimental outcomes in both host and non-host assays. We will consider how some of the effects on important cellular functions linked to these regulatory systems, including production of secondary metabolites, and the overall impact on competition and virulence.

## THE IMPACT OF RESISTANCE MUTATIONS ON BACTERIAL FITNESS AND COMPETITIVE ADVANTAGE IN NON-HOST ENVIRONMENTS

A single point mutation may impose pleiotropic effects on a bacterial system, fundamentally altering bacterial response, metabolism and physiology in a general, non-host environment. Spontaneous mutants have been shown to display altered fitness and growth rate due to the inherent cost of antibiotic resistance (Andersson and Levin 1999) (Fig. 1). For instance, *Escherichia coli* strains with various spontaneous mutations conferring tigecycline resistance exhibit reduced growth rate in Mueller–Hinton broth as compared to wild type (Linkevicius, Sandegren and Andersson 2013). Mutations in eight different genes were shown to lead to tigecycline resistance, including mutations that alter regulation of the tightly regulated tigecycline efflux system, AcrAB (Keeney *et al.* 2008; Spanu *et al.* 2012; Linkevicius, Sandegren and Andersson 2013). MarA is a positive regulator of the AcrAB efflux system, with MarA being regulated by the repressor, MarR (Keeney *et al.* 2008; Spanu *et al.* 2012; Linkevicius, Sandegren and Andersson 2013). Mutations that impair the function of either MarR or the bacterial Lon protease, which normally functions to degrade MarR, increase the concentration of MarA in the cell, thereby increasing AcrAB production and tigecycline efflux (Keeney *et al.* 2008; Linkevicius, Sandegren and Andersson 2013). Other spontaneously resistant mutants included those with mutations in the heptose biosynthetic pathway, which altered lipopolysaccharide maturation and reduced porin production, thus restricting the entry of tigecycline into the cell which is presumed to enter the cell via porins such as OmpF (Spanu *et al.* 2012; Linkevicius, Sandegren and Andersson 2013). An assessment of comparative growth of these mutants relative to wild type showed a cost of fitness as determined by growth rate in culture media (Linkevicius, Sandegren and Andersson 2013). In addition, these lipopolysaccharide mutants are also more susceptible to other antibiotics, unlike the wild-type strain (Linkevicius, Sandegren and Andersson 2013). The *in vitro* global changes that accompany tigecycline resistance

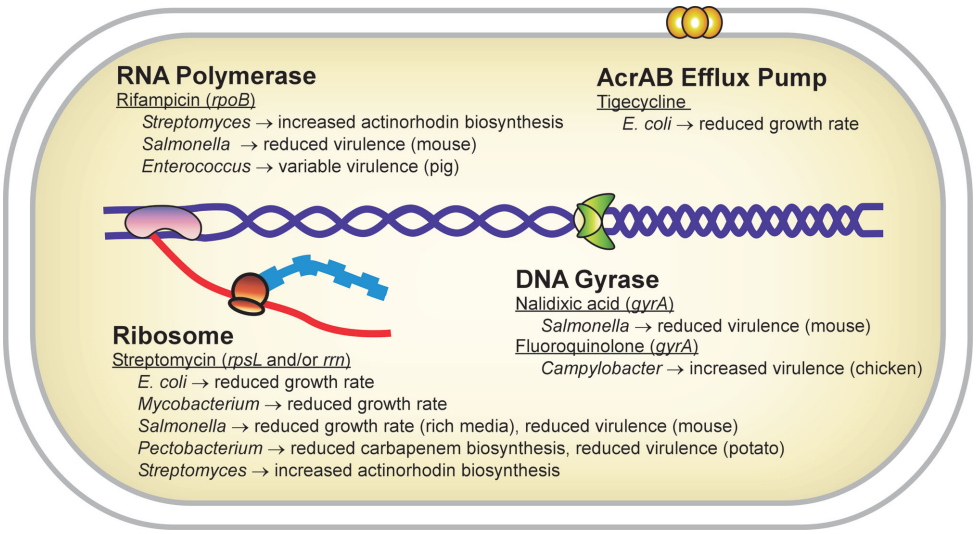


**Figure 1.** Point mutations conferring resistance to some of the most commonly used antibiotic markers, such as rifampicin, tigecycline, streptomycin, nalidixic acid and fluoroquinolone may alter microbial phenotype, including growth rate, virulence and antibiotic production.

acquisition can impair growth rate, and weaken the lipopolysaccharide layer.

Studies evaluating mutations in the *rpsL* gene, which confer resistance to streptomycin, also revealed pleiotropic effects on cells (Poggi, Oliveira de Giuseppe and Picardeau 2010). *Escherichia coli* with spontaneous single nucleotide mutations conferring streptomycin resistance were shown to have reduced growth rates relative to the wild-type strain (Ruusala, Andersson and Ehrenberg 1984). These resistance mutations resulted in hyperaccurate ribosomes with enhanced proofreading functions in the absence of streptomycin, which reduced their efficiency, and thus reduced the overall growth rate of mutants compared to wild type (Ruusala, Andersson and Ehrenberg 1984). Similar results were seen in *Mycobacterium smegmatis,* with L42N and L42T mutations in the *rpsL* gene conferring resistance to streptomycin (Sander *et al.* 2002). Using a competition assay, these mutants were shown to have a 15% reduction in growth as compared to wild type in the absence of the antibiotic (Sander *et al.* 2002). Likewise, the streptomycin resistance mutations A523C, C526T and C522T in the ribosomal *rrn* gene result in a reduction in relative fitness between 6 and 9% in competition assays as compared to wild type (Sander *et al.* 2002). One mutation in the 16S rRNA, G524C, conferred a 30% growth disadvantage as compared to the wild type in the competition assay (Sander *et al.* 2002).

Some studies have shown that the growth of spontaneously resistant mutants varies from wild type depending on environmental conditions, including nutrient availability. Streptomycin-resistant mutants of *Salmonella enterica* having the changes K42N and P90S in ribosomal protein S12 (*rpsL*) exhibit reduced growth on rich media, yet grow faster than wild type on lower carbon media (Paulander, Maisnier-Patin and Andersson 2009). The mechanism underlying this phenotype relates to the mutations impairing capacity to induce the alternate sigma factor RpoS. When carbon is limited, RpoS naturally inhibits the growth of wild-type cells (Paulander, Maisnier-Patin and Andersson 2009). The K42N and P90S mutants are freed of this growth inhibition imposed by RpoS, enabling them to grow faster than wild type under carbon-limited conditions (Paulander, Maisnier-Patin and Andersson 2009). Yet in other cases, point mutations conferring antibiotic resistance may have no effect on growth, such as with *M. smegmatis* mutants carrying the L42R mutation in the *rpsL* gene, which have similar growth rates as the wild type when evaluated by competition assay (Sander *et al.* 2002). In other cases, such as with fluoroquinolone resistance in *E. coli* MG1655, the genetic background in which the mutation arises will determine its fitness costs (Marcusson, Frimodt-Moller and Hughes 2009). Five mutations conferring fluoroquinolone resistance, including *gyrA*1 (S83L), *gyrA*2 (D87N) and *parC* (S80I), along with deletion of *marR* and *arcR,* individually decrease the overall fitness of the microbe, but all five of these mutations together confer resistance without the same reduction in fitness (Marcusson, Frimodt-Moller and Hughes 2009).

In addition to growth rate and fitness, point mutations conferring antibiotic resistance can impact several other phenotypes, including the production of biologically active secondary metabolites. Mutations K43N and K43T in the *rpsL* gene of *Pectobacterium carotovorum* (formerly *Erwinia carotovora*) confer streptomycin resistance; however, these changes result in the loss of carbapenem production (Barnard *et al.* 2010). The *car* genes, which direct the biosynthesis of carbapenem and whose regulation is linked directly to quorum sensing, were expressed at a reduced rate in the mutants as compared to wild type (Barnard *et al.* 2010). Quorum sensing, sometimes called diffusion sensing, is a regulatory mechanism in which bacterial gene expression is dependent upon the environmental concentration of a bacterially derived signalling molecule (Miller and Bassler 2001; Whitehead *et al.* 2001). High bacterial cell densities can result in accumulation of the autoinducer, which in turn causes induction or repression of various gene pathways (Miller and Bassler 2001; Redfield 2002). In the case of *P. carotovorum*, the production of the antibiotic carbapenem as well as production of virulence factors including pectate lysases, cellulases and proteases all appear to be regulated by the N-(3-oxohexanoyl)-L-homoserine lactone (OHHL)-dependent quorum-sensing system (McGowan *et al.* 2005). OHHL-dependent carbapenem production is activated by the *carR, carI* and *hor* genes (McGowan *et al.* 1995; McGowan *et al.* 2005; Sjöblom *et al.* 2008; Bibb and Hesketh 2009). CarI produces the OHHL, which activates CarR, and CarR then activates transcription of the carbapenem biosynthetic cluster (McGowan *et al.* 2005; Sjöblom *et al.* 2008). The product of the *hor* gene, an SlyA/MarR-like transcriptional regulator, is a global activator that is also involved in cluster regulation and activation of the *car pathway*, although its specific roles are still unclear (McGowan *et al.* 2005; Sjöblom *et al.* 2008). Thus, in the streptomycin-resistant mutants, a reduction of carbapenem production was attributed to the *rpsL* mutations reducing levels of the Hor global activator that activates the *car* biosynthetic cluster by an as-yet-identified mechanism (Barnard *et al.* 2010). Carbapenem production can be restored in these mutants through the exogenous addition of OHHL (Barnard *et al.* 2010). This illustrates the global impact of only a single point mutation to overall gene regulation and physiology.

In some cases, spontaneous antibiotic resistance mutants can overproduce or underproduce a secondary metabolite relative to wild type. *Streptomyces coelicolor* produces the blue-pigmented antibiotic, actinorhodin, which was found to compromise phospholipid bilayer integrity in a variety of Gram-positive bacteria (Lakey *et al.* 1983; Hu and Ochi 2001). Actinorhodin production is suppressed by streptomycin resistance mutations in the ppGpp biosynthetic genes, *relA* and *relC* (Hu and Ochi 2001). The changes in actinorhodin expression appear to be linked to its regulation by the stringent response (Hu and Ochi 2001), a regulatory mechanism responsible for the activation of an amino acid starvation response (Chatterji, Fujita and Ishihama 1998; Hesketh *et al.* 2007; Dalebroux *et al.* 2010). During the stringent response, the allosteric regulator ppGpp binds the beta subunit of the RNA polymerase to alter transcription (Chatterji, Fujita and Ishihama 1998; Zuo, Wang and Steitz 2013). This, in turn, causes a reduction in total RNA synthesis, which is generally followed by the upregulation of amino acid biosynthesis and other stress responses (Traxler *et al.* 2008). Mutations that alter K88 of the S12 ribosomal protein were able to restore antibiotic production in *relA* and *relC* mutants, suggesting restoration of actinorhodin production through the induction of a pathway outside of the ppGpp pathway (Hu and Ochi 2001). In fact, actinorhodin production can be upregulated by rifampicin resistance mutations in the beta subunit gene of the RNA polymerase (*rpoB*), as well as streptomycin and gentamycin resistance mutations in *rpsL*, the gene encoding ribosomal protein S12 (Hu and Ochi 2001). It was suggested that these mutations increase production by bypassing the ppGpp binding step, or change the RNA polymerase such that it mimicks the ppGpp-bound form (Hu and Ochi 2001).

In *S. lividans*, the streptomycin-resistant *rpsL* mutants, K88E and P91S, also exhibit an increase in the actinorhodin production, which was shown to be caused by enhanced translation due to increased stability of the ribosome (Okamoto-Hosoya, Hosaka and Ochi 2003). Actinorhodin overproduction was found

to be linked to an increase in ribosomal recycling factor (RRF) (Hosaka, Xu and Ochi 2006). RRF is a translation factor that enhances production of proteins by disassembling the 30s and 50s subunits of the ribosome, increasing protein turnaround and enabling the reinitiation of translation (Hosaka, Xu and Ochi 2006). The increase in RRF was a result of the *rpsL* mutant upregulating the production of *ffr*, the gene that encodes the RRF; however, the mechanism of this regulatory effect is not yet understood (Hosaka, Xu and Ochi 2006).

Point mutations conferring a resistance phenotype can fundamentally alter physiology. Mutations that result in increased ribosome stability and translation initiation, for example, are expected to have broad impact on cellular functions. Mutants may exhibit reduced fitness, or altered anabolic and catabolic capabilities, making them non-representative of the wild-type strain. Such effects should always be taken into consideration for any *in vitro* study.

## THE IMPACT OF RESISTANCE MUTATIONS ON BACTERIAL FITNESS AND VIRULENCE IN HOST ENVIRONMENTS

Many *in vitro* studies have evaluated and quantified the pleiotropic consequences of point mutations conferring antibiotic resistance, and such effects extend to *in vivo* studies as well. The pleiotropic effects of a single nucleotide mutation conferring antibiotic resistance have been shown to render mutants defective in the ability to induce certain virulence factors (Fig. 1). Virulence factors allow for bacteria to survive and multiply within a host, facilitating infection (Kirzinger and Stavrinides 2012). *Pectobacterium carotovorum* mutants that gained spontaneous resistance to streptomycin due to K43T and K43N mutations in the *rpsL* gene were shown to exhibit a significant reduction in virulence towards potato tubers (Barnard *et al.* 2010). A proteomic analysis using 2D difference gel electrophoresis revealed that the *rpsL* mutations resulted in the downregulation of at least 45 proteins, and the upregulation of 55 proteins. The authors proposed that the reduction in virulence was related to changes in expression of the *hor* genes, which are regulators of virulence (Barnard *et al.* 2010). One *rpsL* mutant K43R did not show any decrease in virulence compared to wild type (Barnard *et al.* 2010), indicating that different resistance mutations in the same gene can vary in phenotype from wild type.

Similar results were obtained in studies of virulence of *Sa. enterica* serovar Typhimurium mutants having spontaneous mutations conferring resistance to streptomycin (*rpsL*), rifampicin (*rpoB*) and nalidixic acid (*gyrA*) (Björkman, Hughes and Andersson 1998). The virulence of mutants was compared with that of wild type in an *in vivo* mouse model, wherein bacteria were injected into the host, and following incubation, the spleen harvested and bacterial titres quantified (Björkman, Hughes and Andersson 1998). Mutants showed reduced growth in the mouse, with a doubling time as high as 58 minutes as compared to 26 minutes for wild type (Björkman, Hughes and Andersson 1998).

A point mutation conferring antibiotic resistance can increase the fitness of a microbe in the host. In the chicken pathogen *Campylobacter jejuni,* the fluoroquinolone resistance mutation C257T in *gyrA* confers increased fitness in the chicken model, with mutants not only outcompeting the wild type, but also having an enhanced ability to colonize the host (Luo *et al.* 2005). Although the exact mechanism by which the mutation increases fitness is unknown, it does not appear to be the result of compensatory mutations (Luo *et al.* 2005). Likewise, a study with *Enterococcus faecium* demonstrated varying growth rate and survival of different rifampicin-resistant mutants during colonization of the pig gut (Enne *et al.* 2004). The 12 mutants examined carried diverse mutations in the *rpoB* gene, with the majority of mutations being H489Y/Q (Enne *et al.* 2004). Mutants exhibited anywhere from a 2.5% increase in fitness to a 10% decrease in fitness during colonization of the pig gut as compared to the wild type, with the H489Y/Q mutations conferring increased fitness (Enne *et al.* 2004). The use of such mutants for *in vivo* fitness assays may therefore not fully represent the ability of the wild-type microbe to colonize a host environment.

Similar fitness costs were observed by examining the impact of norfloxacin on the ability of *E. coli* to cause urinary tract infections (Komp Lindgren *et al.* 2005). Resistance to norfloxacin was shown to increase with mutations in *gyrA, gyrB, parC* and *parE* (Komp Lindgren *et al.* 2005); however, these mutations reduced overall fitness in both *in vitro* competition assays with wild type and *in vivo* competition assays in a mouse model (Komp Lindgren *et al.* 2005). Secondary mutations in the *gyrA, gyrB, gyrC, marOR, parC* and *acrR* genes were also identified in mutants, which appeared to partially compensate for the reduction in fitness caused by the initial resistance mutation (Komp Lindgren *et al.* 2005).

## IMPLICATIONS

The pleiotropic effects of single nucleotide mutations conferring antibiotic resistance can be quite dramatic, resulting in significant phenotypic differences between mutants and wild-type strains that can impact both *in vitro* and *in vivo* experiments. Many laboratories use spontaneous antibiotic-resistant mutants of wild-type isolates for host assays and competition experiments, and may neglect to consider the cellular changes that can occur with even a single point mutation. Such mutants can have a substantially divergent phenotype from wild type, including over- or underproduction of secondary metabolites or virulence factors, which may directly influence microbial fitness in the specific environmental condition or niche being evaluated. Because resistance to an antibiotic can arise through multiple mechanisms, phenotypes may vary depending on the gene affected and even between different mutations within that gene. Furthermore, it is important to bear in mind that serial passage of a microbe through either *in vitro* or *in vivo* assay conditions may result in the accumulation of compensatory mutations that offset the costs of the original resistance mutation. Therefore, careful characterization of each independent mutant would be prudent to establish the nature and degree of phenotypic difference, if any, between a spontaneously resistant antibiotic mutant and wild type. The impact of such mutations on global changes in gene expression provides unique opportunities to explore and understand the regulatory networks linked to antibiotic resistance, and may provide essential information on strategies for antibiotic treatment wherein specific antibiotic combinations may slow the evolution of resistance.

There are other alternatives to using spontaneous antibiotic resistance mutants, such as the introduction of antibiotic resistance genes and fluorescent tags into wild-type strains (Kreth *et al.* 2005), yet the system-level impact of heterologous expression on the microbe is not always clear. The use of qPCR or community profiling using 16S rRNA or similar marker can also provide an alternative means for quantification, although these approaches are considerably more expensive and are often not feasible for smaller scale assays. When available, the

use of selective media could be used in host or competition assays to limit the growth of other microbial species, thus eliminating the need to use resistance mutants as a proxy for wild type. Ultimately, *in vivo* and *in vitro* assays are essential to the advancement of our understanding of microbial virulence, gene regulation and general physiology; however, it is critical that researchers carefully consider the use of any resistance mutants in their experimental designs, as these may not always be appropriate proxies for the wild-type isolate.

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## REFERENCES

Andersson DI, Levin BR. The biological cost of antibiotic resistance. *Curr Opin Microbiol* 1999;**2**:489–93.

Aubry-Damon H, Galimand M, Gerbaud G, *et al. rpoB* mutation conferring rifampin resistance in *Streptococcus pyogenes*. *Antimicrob Agents Ch* 2002;**46**:1571–3.

Barnard AML, Simpson NJL, Lilley KS, *et al.* Mutations in *rpsL* that confer streptomycin resistance show pleiotropic effects on virulence and the production of a carbapenem antibiotic in *Erwinia carotovora*. *Microbiology* 2010;**156**:1030–9.

Bibb M, Hesketh A. Analyzing the regulation of antibiotic production in streptomycetes. *Methods Enzymol* 2009;**458**:94–113.

Björkman J, Hughes D, Andersson DI. Virulence of antibiotic-resistant *Salmonella typhimurium*. *P Natl Acad Sci USA* 1998;**95**:3949–53.

Chatterji D, Fujita N, Ishihama A. The mediator for stringent control, ppGpp, binds to the $\beta$-subunit of *Escherichia coli* RNA polymerase. *Gene Cell* 1998;**3**:279–87.

Dalebroux ZD, Svensson SL, Gaynor EC, *et al.* ppGpp conjures bacterial virulence. *Microbiol Mol Biol R* 2010;**74**:171–91.

Enne VI, Delsol AA, Roe JM, *et al.* Rifampicin resistance and its fitness cost in *Enterococcus faecium*. *J Antimicrob Chemoth* 2004;**53**:203–7.

Finken M, Kirschner P, Meier A, *et al.* Molecular basis of streptomycin resistance in *Mycobacterium tuberculosis*: alterations of the ribosomal protein S12 gene and point mutations within a functional 16S ribosomal RNA pseudoknot. *Mol Microbiol* 1993;**9**:1239–46.

Hesketh A, Chen WJ, Ryding J, *et al.* The global role of ppGpp synthesis in morphological differentiation and antibiotic production in *Streptomyces coelicolor* A3(2). *Genome Biol* 2007;**8**:R161.

Hosaka T, Xu J, Ochi K. Increased expression of ribosome recycling factor is responsible for the enhanced protein synthesis during the late growth phase in an antibiotic-overproducing *Streptomyces coelicolor* ribosomal *rpsL* mutant. *Mol Microbiol* 2006;**61**:883–97.

Hu H, Ochi K. Novel approach for improving the productivity of antibiotic-producing strains by inducing combined resistant mutations. *Appl Environ Microb* 2001;**67**:1885–92.

Jin DJ, Gross CA. Mapping and sequencing of mutations in the *Escherichia coli rpoB* gene that lead to rifampicin resistance. *J Mol Biol* 1988;**202**:45–58.

Keeney D, Ruzin A, McAleese F, *et al.* MarA-mediated overexpression of the AcrAB efflux pump results in decreased susceptibility to tigecycline in *Escherichia coli*. *J Antimicrob Chemoth* 2008;**61**:46–53.

Kirzinger MWB, Stavrinides J. Host specificity determinants as a genetic continuum. *Trends Microbiol* 2012;**20**:88–93.

Komp Lindgren P, Marcusson LL, Sandvang D, *et al.* Biological cost of single and multiple norfloxacin resistance mutations in *Escherichia coli* implicated in urinary tract infections. *Antimicrob Agents Ch* 2005;**49**:2343–51.

Kreth J, Merritt J, Shi W, *et al.* Competition and coexistence between *Streptococcus mutans* and *Streptococcus sanguinis* in the dental biofilm. *J Bacteriol* 2005;**187**:7193–203.

Lakey JH, Lea EJA, Rudd BAM, *et al.* A new channel-forming antibiotic from *Streptomyces coelicolor* a3(2) which requires calcium for its activity. *Microbiology* 1983;**129**:3565–73.

Lambert P. Bacterial resistance to antibiotics: modified target sites. *Adv Drug Deliv Rev* 2005;**57**:1471–85.

Linkevicius M, Sandegren L, Andersson DI. Mechanisms and fitness costs of tigecycline resistance in *Escherichia coli*. *J Antimicrob Chemoth* 2013;**68**:2809–19.

Luo N, Pereira S, Sahin O, *et al.* Enhanced *in vivo* fitness of fluoroquinolone-resistant *Campylobacter jejuni* in the absence of antibiotic selection pressure. *P Natl Acad Sci USA* 2005;**102**:541–6.

McGowan S, Sebaihia M, Jones S, *et al.* Carbapenem antibiotic production in *Erwinia carotovora* is regulated by CarR, a homologue of the LuxR transcriptional activator. *Microbiology* 1995;**141**:541–50.

McGowan SJ, Barnard AML, Bosgelmez G, *et al.* Carbapenem antibiotic biosynthesis in *Erwinia carotovora* is regulated by physiological and genetic factors modulating the quorum sensing-dependent control pathway. *Mol Microbiol* 2005;**55**:526–45.

Marcusson LL, Frimodt-Moller N, Hughes D. Interplay in the selection of fluoroquinolone resistance and bacterial fitness. *PLoS Pathog* 2009;**5**:e1000541.

Miller MB, Bassler BL. Quorum sensing in bacteria. *Annu Rev Microbiol* 2001.**55**:165–99.

Okamoto-Hosoya Y, Hosaka T, Ochi K. An aberrant protein synthesis activity is linked with antibiotic overproduction in *rpsL* mutants of *Streptomyces coelicolor* A3(2). *Microbiology* 2003;**149**:3299–309.

Olkkola S, Juntunen P, Heiska H, *et al.* Mutations in the *rpsL* gene are involved in streptomycin resistance in *Campylobacter coli*. *Microb Drug Resist* 2010;**16**:105–10.

Paulander W, Maisnier-Patin S, Andersson DI. The fitness cost of streptomycin resistance depends on *rpsL* mutation, carbon source and RpoS (sigmaS). *Genetics* 2009;**183**:539–46.

Poggi D, Oliveira de Giuseppe P, Picardeau M. Antibiotic resistance markers for genetic manipulations of *Leptospira spp*. *Appl Environ Microb* 2010;**76**:4882–5.

Redfield R. Is quorum sensing a side effect of diffusion sensing? *Trends Microbiol* 2002;**10**:365–70.

Ruusala T, Andersson D, Ehrenberg M. Hyper-accurate ribosomes inhibit growth. *EMBO J* 1984;**3**:2575–80.

Sander P, Springer B, Prammananan T, *et al.* Fitness cost of chromosomal drug resistance-conferring mutations. *Antimicrob Agents Ch* 2002;**46**:1204–11.

Sjöblom S, Harjunpää H, Brader G, *et al.* A novel plant ferredoxin-like protein and the regulator Hor are quorum-sensing targets in the plant pathogen *Erwinia carotovora*. *Mol Plant Microbe In* 2008;**21**:967–78.

Spanu T, De Angelis G, Cipriani M, *et al*. *In vivo* emergence of tigecycline resistance in multidrug-resistant *Klebsiella pneumoniae* and *Escherichia coli*. *Antimicrob Agents Ch* 2012;**56**: 4516–8.

Springer B, Kidan YG, Prammananan T, *et al*. Mechanisms of streptomycin resistance: selection of mutations in the 16S rRNA gene conferring resistance. *Antimicrob Agents Ch* 2001;**45**:2877–84.

Sreevatsan S, Pan X, Stockbauer KE, *et al*. Characterization of *rpsL* and *rrs* mutations in streptomycin-resistant *Mycobacterium tuberculosis* isolates from diverse geographic localities. *Antimicrob Agents Ch* 1996;**40**:1024–6.

Traxler MF, Summers SM, Nguyen HT, *et al*. The global, ppGpp-mediated stringent response to amino acid starvation in *Escherichia coli*. *Mol Microbiol* 2008;**68**:1128–48.

Whitehead NA, Barnard AM, Slater H, *et al*. Quorum-sensing in Gram-negative bacteria. *FEMS Microbiol Rev* 2001;**25**: 365–404.

Yoshida H, Bogaki M, Nakamura M, *et al*. Quinolone resistance-determining region in the DNA gyrase gyrA gene of *Escherichia coli*. *Antimicrob Agents Ch* 1990;**34**:1271–2.

Zuo Y, Wang Y, Steitz TA. The mechanism of *E. coli* RNA polymerase regulation by ppGpp is suggested by the structure of their complex. *Mol Cell* 2013;**50**:430–6.