The Fitness Cost of Rifampicin Resistance in *Pseudomonas aeruginosa* Depends on Demand for RNA Polymerase

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ABSTRACT

Bacterial resistance to antibiotics usually incurs a fitness cost in the absence of selecting drugs, and this cost of resistance plays a key role in the spread of antibiotic resistance in pathogen populations. Costs of resistance have been shown to vary with environmental conditions, but the causes of this variability remain obscure. In this article, we show that the average cost of rifampicin resistance in the pathogenic bacterium *Pseudomonas aeruginosa* is reduced by the addition of ribosome inhibitors (chloramphenicol or streptomycin) that indirectly constrain transcription rate and therefore reduce demand for RNA polymerase activity. This effect is consistent with predictions from metabolic control theory. We also tested the alternative hypothesis that the observed trend was due to a general effect of environmental quality on the cost of resistance. To do this we measured the fitness of resistant mutants in the presence of other antibiotics (ciprofloxacin and carbenicillin) that have similar effects on bacterial growth rate but bind to different target enzymes (DNA gyrase and penicillin-binding proteins, respectively) and in 41 single-carbon source environments of varying quality. We find no consistent effect of environmental quality on the average cost of resistance in these treatments. These results show that the cost of rifampicin resistance varies with demand for the mutated target enzyme, rather than as a simple function of bacterial growth rate or stress.

CHROMOSOMAL antibiotic resistance evolves by mutations that modify the structure of enzymes that play key roles in cellular physiology (WALSH 2000; ANDERSSON 2003; MAISNIER-PATIN and ANDERSSON 2004), and it is therefore not surprising that resistance mutations tend to incur a fitness cost that is expressed as a reduction in competitive ability, transmission rate, and virulence (ANDERSSON 2006). Epidemiological studies have shown that this cost of resistance plays a key role in determining the spread of resistance in pathogen populations, and it has even been suggested that the cost of resistance is the single most important driver of resistance evolution in pathogen populations (ANDERSSON and HUGHES 2010). Understanding the factors that determine the cost of antibiotic resistance is therefore key to our overall understanding of the evolution of antibiotic resistance in bacterial pathogens. Experimental studies have shown that the cost of resistance, even for the same mutation, varies as a result of environmental conditions (BJÖRKMAN *et al.* 2000; NAGAEV *et al.* 2001; PAULANDER *et al.* 2009). For example, the relative fitness of *Salmonella typhimurium* mutants resistant to streptomycin

or fusidic acid is different in mice and *in vitro* (BJÖRKMAN *et al.* 2000). However, the mechanisms that create variation in costs of resistance are not understood (for an exception, see PAULANDER *et al.* 2009), despite a detailed understanding of the molecular basis of antibiotic resistance. Given that chromosomal resistance mutations compromise the function of essential genes, a simple explanation for environmental variation of the cost of resistance is that different environments impose different levels of demand for activity of the mutated target enzyme. To test this hypothesis, we measured the fitness cost of rifampicin resistance mutations across environments that impose variable levels of demand for the mutated enzyme (RNA polymerase).

Rifampicin works by binding to a highly conserved pocket on the β -subunit of RNA polymerase and blocking RNA transcript elongation (SEVERINOV *et al.* 1993; CAMPBELL *et al.* 2001). Resistance results from mutations on the *rpoB* gene that change the structure of the binding pocket and block antibiotic-target binding (TELENTI *et al.* 1993; POZZI *et al.* 1999; CAMPBELL *et al.* 2001; TRINH *et al.* 2006). To estimate the contribution of demand for RNA polymerase to the cost of resistance in rifampicin-free environments, we measured the fitness of 53 rifampicin-resistant mutants of *Pseudomonas aeruginosa* across a range of environments where we experimentally manipulated demand for RNA polymerase by adding sublethal doses of ribosomal inhibitors. The

Supporting information is available online at <http://www.genetics.org/cgi/content/full/genetics.110.124628/DC1>.

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rationale for this manipulation is provided by metabolic control theory: gene expression and protein synthesis can be seen as a linear pathway, and metabolic control theory predicts how flux through this pathway responds to perturbations of individual enzymes (MACLEAN 2010). In a pathway made up of n unsaturated enzymes, the control of flux through the pathway exerted by the i th enzyme can be described by a control coefficient, c_i , that describes how flux through the pathway, J_P , varies as a result of the activity of the enzyme, E_i , such that $c_i = dJ_P/dE_i$. A fundamental property of such pathways is that the sum of all control coefficients is always equal to 1 (KLIPP *et al.* 2009), and this law of conservation of the control of flux implies that increasing the control of flux exerted by one enzyme by reducing its activity will necessarily reduce the control on flux exerted by other enzymes in the pathway (SZATHMÁRY 1993; MACLEAN 2010). For example, if the rate of protein synthesis is limited exclusively by the rate of translation (*i.e.*, $c_i = 1$), then environmental or genetic perturbations that lead to mild or moderate reductions in the rate of transcription will have no effect on the overall rate of protein synthesis. Recent experiments by PROSHKIN *et al.* (2010) support this theoretical argument: bacterial transcription and translation rates are closely coupled, and the addition of ribosomal inhibitors indirectly reduces transcription rate, effectively placing a speed limit on RNA polymerase activity and implying reduced demand for this enzyme under ribosomal inhibition. We therefore predicted that the average cost of rifampicin resistance mutations on RNA polymerase would be smaller in the presence of ribosomal inhibitors.

An alternative explanation that has been put forward to explain variation in the fitness costs of deleterious mutations in general (SHABALINA *et al.* 1997; KORONA 1999; SZAFRANIEC *et al.* 2001), including antibiotic resistance mutations (PETERSEN *et al.* 2009), is that fitness costs are amplified in stressful conditions or poor-quality environments. This is an important alternative hypothesis, because demand for RNA polymerase cannot be manipulated by ribosomal inhibition without imposing a reduction in bacterial growth rate. To disentangle the effects of ribosomal inhibition and environmental quality, measured as growth rate of the wild type, we therefore used two approaches. First, we measured the fitness cost of rifampicin resistance in the presence of growth inhibitors that target cellular processes that are not directly involved in gene expression and protein synthesis (DNA supercoiling and cell wall assembly). Second, we estimated the cost of resistance across a wide range of single carbon source environments, testing for a correlation between environmental quality and the cost of resistance.

We show that experimentally reducing demand for RNA polymerase using ribosomal inhibitors reduces the average cost of rifampicin resistance and that this manipulation can even completely eliminate the cost of

resistance. In contrast, other types of growth inhibition have no consistent effect on the cost of resistance: other antibiotics or poor-quality environments can either aggravate or alleviate the cost of rifampicin resistance. We conclude that enzyme demand may provide a general explanation for environmental variability in the cost of resistance and we argue that this relationship could be used to contribute to the design of treatment strategies for minimizing the spread of resistance in pathogen populations.

MATERIALS AND METHODS

Bacterial genotypes: Rifampicin-resistant genotypes of *P. aeruginosa* PA01 each carried either one or two amino acid substitutions on *rpoB*, totaling 13 single and 40 double mutants (supporting information, Table S1). Single mutants were isolated from a fluctuation test with the rifampicin-sensitive wild-type *P. aeruginosa* PA01 on agar plates containing $62.11 \mu\text{g mL}^{-1}$ rifampicin (MACLEAN and BUCKLING 2009). Double mutants were isolated by selecting three genotypes carrying low levels of rifampicin resistance for high levels of resistance (MACLEAN *et al.* 2010). Mutations on *rpoB* were identified by sequencing the central rifampicin resistance region, as described in detail in MACLEAN and BUCKLING (2009).

Fitness assays: We measured fitness as growth rate relative to wild-type PA01 at 37° in M9KB media (MACLEAN and BUCKLING 2009). For each assay, $1 \mu\text{l}$ of overnight culture was added to $200 \mu\text{l}$ of M9KB in a randomly assigned well of a 96-well microplate and OD_{600} measured by spectrophotometry every hour for 12 hr. Growth rate during the exponential growth phase was then estimated using Gen5 software (BioTek Instruments). Each assay was replicated three or four times in each block. For assays in the presence of antibiotics, we assayed fitness (growth rate relative to that of the wild type measured in the same conditions) at three concentrations of each drug: 0, 1.05, and 4.2 mg/liter chloramphenicol; 0, 15, and 30 mg/liter streptomycin; 0, 7, and 28 mg/liter carbenicillin; and 0, 12, and $24 \mu\text{g/liter}$ ciprofloxacin. These concentrations were chosen to cause a similar reduction in bacterial growth in each treatment (Figure S1; File S1). Assays for different antibiotics were conducted on different days; we therefore assayed all genotypes in the absence of antibiotics in each block of assays, finding that replicate measurements in separate blocks were strongly correlated with each other ($r = 0.73$ on average).

Biolog assays: To test for a general relationship between environmental quality and the cost of rifampicin resistance, we estimated the fitness of 24 of the mutants described above in each of the 95 environments represented by the different carbon substrates on Biolog (Hayward, CA) GN2 microplates (A. R. HALL and R. C. MACLEAN, unpublished results). To assay a given genotype, we diluted $60 \mu\text{l}$ of overnight culture in 17 ml of M9 solution, starved the cells for at least 2 hr, and then added $150 \mu\text{l}$ to each well of a Biolog plate and incubated it for 24 hr at 37° . Due to the large number of Biolog assays ($n = 7200$), we were unable to record growth rates over time and instead estimated fitness from OD_{660} after 24 hr of growth (MACLEAN and BELL 2002). This measure is strongly positively correlated with exponential growth rates of the wild type across all Biolog substrates ($r^2 = 0.82$; A. R. HALL and R. C. MACLEAN, unpublished results). OD_{660} scores were corrected by subtracting the score for the control well (water), and we took the ratio of OD_{660} relative to OD_{660} for the wild type as an estimate of fitness for each combination of genotype and

substrate; each assay was replicated three times. We restricted our analysis to the 41 substrates where the wild type showed positive growth ($OD_{660} > 0.15$).

Statistical analyses: We tested for variation in absolute growth rate and relative fitness using mixed effects models with antibiotic concentration as a categorical factor and genotype as a random effect. We tested whether resistance incurred a fitness cost on average at each concentration by testing the distribution of fitness scores against a mean of 1.0 at each concentration, adjusting significance levels by sequential Bonferroni correction to account for multiple comparisons within each antibiotic treatment.

RESULTS

Rifampicin resistance carries a fitness cost: To test for a fitness cost associated with rifampicin resistance, we measured the growth rates of 53 strains of *P. aeruginosa* each carrying either one or two rifampicin resistance mutations in *rpoB* in nutrient-rich culture medium lacking antibiotics. Consistent with previous studies, we found that the average fitness of resistant strains was less than that of the wild type (mean $\pm$ SD = 0.88 ± 0.21 ; $t_{52} = 4.08$, $P < 0.001$), directly demonstrating a cost of rifampicin resistance that varied between genotypes. Mean fitness did not differ significantly between single and double mutants (mean $\pm$ SE = 0.88 ± 0.07 , $N = 13$ for single mutants; 0.88 ± 0.03 , $N = 40$ for double mutants; Welch's $t_{17.2} = 0.06$, $P = 0.95$).

Reducing demand for RNA polymerase eliminates the cost of rifampicin resistance: To manipulate demand for RNA polymerase, we added sublethal doses of chloramphenicol and streptomycin, antibiotics that inhibit ribosomal activity and therefore indirectly control transcription rate (RUUSALA *et al.* 1984; PROSHKIN *et al.* 2010). The average fitness cost of rifampicin resistance was reduced under increasing doses of both ribosome inhibitors (chloramphenicol: $F_{2, 104} = 16.69$, $P < 0.001$; streptomycin: $F_{2, 104} = 4.23$, $P = 0.017$; Figure 1). Specifically, sublethal doses of either ribosome inhibitor eliminated the average cost of rifampicin resistance: the mean fitness of resistant genotypes was not significantly different from the wild type at the highest concentrations of chloramphenicol ($t_{52} = 1.90$, $P = 0.06$; Figure 1A) and streptomycin ($t_{52} = 0.09$, $P = 0.92$; Figure 1B). The increase in fitness under ribosome inhibition was greatest for those genotypes that had large fitness costs in antibiotic-free medium (chloramphenicol: $F_{1, 51} = 43.55$, $P < 0.0001$; streptomycin: $F_{1, 51} = 8.82$, $P = 0.005$; Figure S2).

The cost of resistance is not elevated in poor-quality environments: We tested whether the reduced cost of resistance in chloramphenicol and streptomycin treatments was specific to ribosome inhibitors, or due to a general effect of growth inhibition on fitness costs, by two different experiments. First, we measured the fitness of each mutant in the presence of antibiotics that inhibit either DNA supercoiling (ciprofloxacin) or cell wall assembly (carbenicillin); these antibiotics were

FIGURE 1.—Costs of rifampicin resistance in the presence of ribosome inhibitors. Fitness of rifampicin-resistant mutants, measured as growth rate relative to the wild type (1.0) at 37°C, is shown at three concentrations of (A) chloramphenicol and (B) streptomycin. Bars, $\pm$ SE for 53 mutants.

added at equivalent doses to the ribosomal inhibitors above (Figure S1). The addition of either antibiotic had a significant effect on the cost of resistance (carbenicillin: $F_{2, 104} = 51.04$, $P < 0.0001$; ciprofloxacin: $F_{2, 104} = 7.37$, $P = 0.001$; Figure 2). However, carbenicillin had the opposite effect to that observed for ribosome inhibitors: the cost of resistance was greatest at the highest concentration (Figure 2A). With the addition of ciprofloxacin, the fitness of rifampicin-resistant mutants was greater than in the absence of antibiotics, but was still significantly less than that of the wild type at both concentrations (12 μ g/liter: $t_{52} = 2.21$, $P = 0.03$; 24 μ g/liter: $t_{52} = 6.36$, $P < 0.0001$; Figure 2B).

As a second test for a general relationship between growth inhibition and the cost of resistance, we tested for a correlation between environmental quality, measured as growth of the wild type across 41 different single carbon source environments, and the average cost of resistance in each environment. Across single carbon source environments, there was no correlation between environmental quality and the average cost of having one or two rifampicin resistance mutations ($F_{1, 39} = 0.0001$, $P = 0.99$; Figure 3). In other words, there was no general tendency for poor environmental conditions to exacerbate fitness costs. Thus, while ribosome inhibitors eliminated the average cost of rifampicin resistance mutations on *rpoB*, there was no consistent effect of growth inhibition caused by other antibiotics or by nutrient limitation.

FIGURE 2.—Costs of rifampicin resistance in the presence of other antibiotics. Fitness of rifampicin-resistant mutants relative to the wild type (1.0) is shown at three concentrations of (A) carbenicillin and (B) ciprofloxacin. Bars, $\pm$ SE for 53 mutants.

DISCUSSION

In summary, we have shown that environmental variation of the cost of rifampicin resistance is driven by demand for RNA polymerase, and not by a general effect of growth inhibition. This conclusion is supported by both a reduction in the cost of rifampicin resistance in environments where demand for RNA polymerase was experimentally reduced and by the lack of an overall tendency for poor quality environments to elevate the cost of resistance.

In economics, the value of a commodity depends on the balance between supply and demand for the commodity in the economy. In the context of a bacterium, the function of an enzyme that is mutated to confer antibiotic resistance can be considered as a commodity in the cellular economy. Our results show that the value of that commodity (its effect on fitness) varies with demand for its function (in this case, RNA polymerase activity). Although we have focused on environmental variation of enzyme demand, it is important to emphasize that demand for enzyme activity may also be determined by the genetic context in which resistance evolves. For example, streptomycin resistance mutations may incur a fitness cost in the absence of antibiotics due to impaired ribosomal function, which may in turn alleviate the fitness cost due to mutations on RNA polymerase by the same mechanism that we observed for ribosomal inhibition in the presence of antibiotics. This is sup-

FIGURE 3.—Average cost of rifampicin resistance across Biolog substrates. Each point shows the average cost of resistance for 24 rifampicin-resistant mutants in 1 of 41 different single carbon source environments. We took growth of wild-type *P. aeruginosa* PA01 as an indicator of environmental quality.

ported by the work of TRINDADE *et al.* (2009), who showed that there is a strong tendency for antagonistic epistasis between antibiotic resistance mutations in the ribosome and RNA polymerase. This finding is entirely consistent with our results and with the principles of metabolic control theory: acquiring deleterious mutations in one enzyme in a pathway reduces demand for other enzymes in the pathway (SZATHMÁRY 1993; MACLEAN 2010); as a result, the combined cost of acquiring multiple mutations is less than that predicted by an additive model (antagonistic epistasis).

The cost of rifampicin resistance was also reduced by the addition of ciprofloxacin, although to a lesser extent than under ribosome inhibition. This result is in agreement with evidence that rifampicin resistance can indirectly confer marginal resistance to DNA gyrase inhibition (BLANC-POTARD *et al.* 1995), meaning that some of our *rpoB* mutants were less susceptible to ciprofloxacin than the wild type. We emphasize that rifampicin resistance was still costly on average at both concentrations of ciprofloxacin.

If the costs of resistance are greatest under environmental and genetic conditions that impose the highest demand for the native function of a mutated protein, it follows that the costs of resistance will also be relatively high for resistance mutations that cause the greatest reduction in enzyme supply (loss of enzyme function). For example, REYNOLDS (2000) demonstrated that the direct effect of rifampicin resistance mutations is to reduce transcription rate and that resistance mutations that lead to the greatest reduction in transcription rate are associated with the greatest fitness cost.

However, it is important to emphasize that rifampicin resistance mutations can have indirect effects that are mediated by pleiotropic changes in the expression of genes that are not related to transcriptional activity; for example, rifampicin resistance mutations lead to changes in the expression of genes involved in metabolism in *Escherichia coli* and sporulation in *Bacillus subtilis* (RYU

1978; JIN and GROSS 1989; PERKINS and NICHOLSON 2008). The fact that reducing demand for RNA polymerase activity can completely eliminate the cost of resistance implies that the cost of resistance stems primarily from the direct effects of rifampicin resistance mutations on RNA polymerase activity. However, several lines of evidence suggest that indirect effects also had important fitness consequences in our experiment. First, we found variation in fitness among rifampicin-resistant mutants in environments where demand for RNA polymerase activity was low, suggesting that indirect effects of resistance mutations contribute to the cost of resistance. Second, the widespread variation in the cost of resistance among environments lacking antibiotics (Biolog substrates) may be due partly to variation in the costs and benefits associated with the pleiotropic effects of rifampicin resistance mutations. In the future, we will attempt to directly determine the contribution to the cost of resistance stemming from direct and indirect effects of resistance mutations.

In short, we argue that an economic model in which the cost of resistance varies with enzyme supply and demand provides a solid theoretical framework for understanding genetic and environmental influences on the average cost of resistance. Given the pivotal importance of fitness costs in determining the spread of resistance in pathogen populations, this suggests that antibiotic therapies that maximize demand for genes involved in resistance may be a novel approach to constraining the evolution of resistance. For example, rifampicin resistance evolution could hypothetically be constrained by administering rifampicin alongside quorum signals that induce the expression of the large number of quorum-regulated genes in the *P. aeruginosa* genome (WHITELEY *et al.* 1999; MILLER and BASSLER 2001), thereby elevating demand for RNA polymerase and increasing the cost of rifampicin resistance.

More generally, this economic model can be extended to understand the fitness effects of other types of deleterious mutations. For example, it has previously been argued that the fitness costs associated with deleterious mutations are linked to environmental stress (SHABALINA *et al.* 1997; KORONA 1999; SZAFRANIEC *et al.* 2001), although the empirical evidence to support this argument is controversial (JASNOS *et al.* 2008). Our work shows that growth inhibition can either aggravate or buffer the fitness effects of deleterious mutations and highlights the importance of integrating molecular information on how physiological perturbations interact with deleterious mutations at a mechanistic level. For example, KISHONY and LEIBLER (2003) showed that stresses that compromise specific cellular targets, such as antibiotics, tend to alleviate the cost of random deleterious mutations whereas general stressors, such as pH, temperature, or osmolarity, have no consistent effect on fitness costs. In the context of our work, these results can be interpreted as follows: targeted sources of stress make

cellular growth limited by a single process (for example, limiting growth by inhibiting the ribosome makes growth rate highly dependent on translation rate), and this effectively reduces demand for the normal functioning of other cellular processes, buffering against the deleterious effects of random mutations.

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Supporting Information

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FIGURE S1.—Bacterial growth rates in different antibiotic treatments. The change in bacterial growth rate was statistically significant in each case (A, chloramphenicol: $F_{2,104} = 26.35$, $P < 0.0001$; B, streptomycin: $F_{2,104} = 177.08$, $P < 0.0001$; C, carbenicillin: $F_{2,104} = 39.25$, $P < 0.0001$; D, ciprofloxacin: $F_{2,104} = 159.56$, $P < 0.0001$).

A

B

C

D

FIGURE S2.—Relative fitness of individual mutants in different antibiotic treatments. Each line shows fitness, measured as growth rate relative to wild type PA01 in the same conditions, for one of 53 different rifampicin-resistant genotypes across three concentrations of (A) chloramphenicol, (B) streptomycin, (C) carbenicillin, (D) ciprofloxacin.

FILE S1**Supporting Data**

File S1 is available for download as a compressed file (.zip) at <http://www.genetics.org/cgi/content/full/genetics.110.124628/DC1>.

TABLE S1
Rifampicin-resistant mutants used in this study

Nucleotide changes		Amino acid changes		Source
1	2	1	2	
A1553G	none	Q518R	none	<i>a</i>
A1553T	C1550T	Q518L	S517L	<i>b</i>
A1553T	none	Q518L	none	<i>a</i>
A1562T	A455G	D521V	Q152R	<i>b</i>
A1562T	none	D521V	none	<i>a</i>
A1592G	none	H531R	none	<i>a</i>
A455G	A1562G	Q152R	D521G	<i>b</i>
A455G	C1550T	Q152R	S517L	<i>b</i>
A455G	C1563A	Q152R	D521E	<i>b</i>
A455G	C1591G	Q152R	H531D	<i>b</i>
A455G	C1593A	Q152R	H531Q	<i>b</i>
A455G	C1736T	Q152R	S579F	<i>b</i>
A455G	G1724C	Q152R	G575A	<i>b</i>
A455G	none	Q152R	none	<i>a</i>
A455G	T1529C	Q152R	F510S	<i>b</i>
A455G	T1547C	Q152R	L516P	<i>b</i>
A455G	T1549G	Q152R	S517A	<i>b</i>
A455G	T449C	Q152R	V150A	<i>b</i>
A455T	A1553G	Q152L	Q518R	<i>b</i>
A455T	A1562G	Q152L	D521G	<i>b</i>
A455T	A1567G	Q152L	N523D	<i>b</i>
A455T	A1592T	Q152L	H531L	<i>b</i>
A455T	A1718G	Q152L	N573S	<i>b</i>
A455T	C1550T	Q152L	S517L	<i>b</i>
A455T	C1563G	Q152L	D521E	<i>b</i>
A455T	C1591T	Q152L	H531Y	<i>b</i>
A455T	C1607T	Q152L	S536F	<i>b</i>
A455T	C1736T	Q152L	S579F	<i>b</i>
A455T	C2296A	Q152L	Q766K	<i>b</i>
A455T	none	Q152L	none	<i>a</i>
A455T	T1549G	Q152L	S517A	<i>b</i>
A455T	T1731C	Q152L	I577T	<i>b</i>
A455T	T1731C	Q152L	I577T	<i>b</i>
C1542G	none	S514R	none	<i>c</i>
C1550T	A1553G	S517L	Q518R	<i>b</i>
C1550T	A1562G	S517L	D521G	<i>b</i>
C1550T	C1375T	S517L	R459C	<i>b</i>
C1550T	C1563G	S517L	D521E	<i>b</i>
C1550T	C1591T	S517L	H531Y	<i>b</i>
C1550T	C1607T	S517L	S536F	<i>b</i>
C1550T	C1610T	S517L	A537V	<i>b</i>
C1550T	C1619T	S517L	P540L	<i>b</i>
C1550T	C1736T	S517L	S579F	<i>b</i>

C1550T	G1561A	S517L	D521N	<i>b</i>
C1550T	G1622A	S517L	G541D	<i>b</i>
C1550T	G1624T	S517L	G542C	<i>b</i>
C1550T	none	S517L	none	<i>a</i>
C1550T	T1655G	S517L	V552G	<i>b</i>
C1563G	none	D521E	none	<i>a</i>
C1591T	A1592G	H531Y	H531R	<i>d</i>
C1591T	none	H531Y	none	<i>a</i>
C1607A	none	S536Y	none	<i>c</i>
C1607T	none	S536F	none	<i>a</i>

a. MacLean & Buckling (2009).

b. MacLean et al. (2010).

c. Fluctuation test using the same protocol as described in MacLean & Buckling (2009).

d. Hall et al. (2010). These substitutions combine to give a single amino acid change (H531C) relative to the wild type.