Development of macrolide resistance in *Bordetella bronchiseptica* is associated with the loss of virulence

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**Background:** Why resistance to specific antibiotics emerges and spreads rapidly in some bacteria confronting these drugs but not others remains a mystery. Resistance to erythromycin in the respiratory pathogens *Staphylococcus aureus* and *Streptococcus pneumoniae* emerged rapidly and increased problematically. However, resistance is uncommon amongst the classic *Bordetella* species despite infections being treated with this macrolide for decades.

**Objectives:** We examined whether the apparent progenitor of the classic *Bordetella* spp., *Bordetella bronchiseptica*, is able to rapidly generate de novo resistance to antibiotics and, if so, why such resistance might not persist and propagate.

**Methods:** Independent strains of *B. bronchiseptica* resistant to erythromycin were generated in vitro by successively passaging them in increasing subinhibitory concentrations of this macrolide. Resistant mutants obtained were evaluated for their capacity to infect mice, and for other virulence properties including adherence, cytotoxicity and induction of cytokines.

**Results:** *B. bronchiseptica* rapidly developed stable and persistent antibiotic resistance de novo. Unlike the previously reported trade-off in fitness, multiple independent resistant mutants were not defective in their rates of growth in vitro but were consistently defective in colonizing mice and lost a variety of virulence phenotypes. These changes rendered them avirulent but phenotypically similar to the previously described growth phase associated with the ability to survive in soil, water and/or other extra-mammalian environments.

**Conclusions:** These observations raise the possibility that antibiotic resistance in some organisms results in trade-offs that are not quantifiable in routine measures of general fitness such as growth in vitro, but are pronounced in various aspects of infection in the natural host.

**Introduction**

On exposure to sublethal levels of antibiotics, some bacteria are known to readily develop antibiotic resistance de novo.¹,² Once resistance has emerged it can spread and amplify especially rapidly in antibiotic-treated hosts that are relatively free of natural competitors. Thus, we observe outbreaks of resistance in environments in which antibiotic use is prevalent, such as amongst patients and staff in hospitals and amongst farm animals mass treated with antibiotics.³,⁴ The potential speed and progress of this process is demonstrated by the case of erythromycin, the prototypical macrolide. Erythromycin binds 23S rRNA, inhibiting protein synthesis by affecting the exit of nascent peptides,⁵ and was hailed as a wonder drug. But within a few years of its first commercial use in 1952, reports of erythromycin-resistant *Staphylococcus aureus* and *Streptococcus pneumoniae* surfaced.⁶ Since then, resistant strains have increased in prevalence and dispersed globally, contributing to a worldwide problem of increasing concern.⁶,⁷ The highly publicized pattern by which antibiotic resistance rapidly evolves and disseminates⁸ has been followed by several important pathogens, but not by others. The *Bordetella* species are...
one of the most prominent of these exceptions. Although erythromycin has been the drug of choice for the treatment of whooping cough (pertussis) for decades, compared with the wide prevalence of resistant S. aureus or S. pneumoniae, large-scale resistance in Bordetella pertussis has been historically rare and continues to be so with the exception of a more recent outbreak reported in northern China. Similarly, the apparent progenitor of the classic Bordetella spp., Bordetella bronchiseptica, which causes respiratory infections in a wide range of mammals (including humans) and birds and is similarly erythromycin treated en masse, has been and largely remains susceptible to erythromycin, demonstrating a counter-example to the rapid rise of erythromycin resistance in Staphylococcus, and suggesting that the relative failure to rapidly evolve and disperse resistance is common to, and may be related to shared characteristics of, these species.

Although the observation that some pathogens rapidly evolve resistance to antibiotics and disseminate widely has been extensively studied as a worrisome example of evolution in real time, the fact that other pathogens do not has garnered less attention. One compelling explanation has been that there is a trade-off: de novo acquired resistance is linked to a loss of fitness, measured as a decreased growth rate in vitro. However, the generality of this trade-off is speculative and its relevance to the specific case of the Bordetella spp. is unknown.

Therefore, the low prevalence of resistance among Bordetella spp. remains enigmatic. From in vitro culture in the presence of increasing sub-MIC concentrations of erythromycin we observed that B. bronchiseptica can rapidly evolve resistance to this macrolide. As measured by their growth rates in vitro, no fitness costs were detected among independent resistant strains isolated in this way, and the high levels of resistance of these strains were maintained through multiple passages (many generations) in the absence of erythromycin. By examining their various characteristics in vitro and in vivo we observed that erythromycin-resistant strains were severely defective in a variety of virulence-associated characteristics. All of these erythromycin-resistant strains were defective in their expression of virulence-associated antigens, and all failed to successfully colonize and persist in the respiratory tracts of their hosts. These results suggest that erythromycin resistance can be readily generated de novo by Bordetella spp. but at a cost to their ability to infect their animal hosts.

Materials and methods

Microbiology

Growth and culture of bacteria

Liquid cultures of B. bronchiseptica strain RB50 and derivative mutants RB54 and RB50i were grown in Stainer Scholte medium (SS medium) supplemented with 0.5% (w/v) heptakis(2,6-di-O-methyl)-β-cyclodextrin (Sigma H0513). Liquid cultures were plated on Bordet Gengou (BG) agar supplemented with 10% (v/v) defibrinated sheep blood (Hemostat, Hemostat Laboratories, USA) and streptomycin (20 mg/L).

Development of resistance and MIC determination

Development of erythromycin resistance in B. bronchiseptica RB50 was assessed starting with four replicate cultures (lineages) of ~10^6 cfu of RB50 grown in 96-well microtitre plates with 200 µL of SS medium per well and 2-fold diluted gradients of erythromycin (0.625–20 mg/L). Bacteria were incubated statically at 37°C for 2 days in a humidified incubator with 5% CO₂. Growth was assessed by OD₆₀₀ readings using a microtitre plate reader. A 10 µL portion of each bacterial culture replicate exceeding an OD₆₀₀ value of 0.5 was then used to seed a fresh erythromycin-gradient microtitre plate containing 190 µL of SS medium per well. Growth in the second plate (passage 2 (p2)) was recorded after 2 days and further passages. Erythromycin concentration gradients for later passages were increased to accommodate growing resistance. MIC ranges of erythromycin (Sigma P1016) for B. bronchiseptica (RB50 (WT), RB54, RB50i and antibiotic-resistant strains) were conducted in microtitre plates using 100 µL of SS medium containing 2-fold dilutions of the antibiotic, at the appropriate concentration ranges. Inhibitory ranges were estimated by identifying the two concentrations of antibiotic between which bacterial cells showed the largest decrease in OD₆₀₀ after 20 h of growth at 37°C.

Mouse experiments

Mouse experiments were performed following recommendations from the Guide for the Care and Use of Laboratory Animals of the NIH. Protocols were approved by the Institutional Animal Care and Use Committee at the University of Georgia, Athens, GA, USA (A2016-02-010-Y2-A3 ‘Bordetella-Host Interactions’). Mice were anaesthetized using 5% isoflurane and euthanized by carbon dioxide inhalation followed by cervical dislocation. Experiments were conducted on 5-week-old female C57BL/6 mice obtained from the Jackson Laboratory, Bar Harbor, ME, USA; for the duration of the experiments, the mice were housed in groups of three or four in home cages at the Specific Pathogen Free facilities of the Central Animal Facility, University of Georgia.

Inoculation and harvesting

Mice were inoculated intranasally by pipetting 5 µL of PBS containing 150 cfu (1 × standard dose) of bacteria into the nares of mice lightly anaesthetized with isoflurane/oxygen. To test colonization by the antibiotic-resistant mutants, 1500 cfu (10×) and 15000 cfu (100×) of bacteria were used to inoculate groups of three or four mice. Bacterial numbers in the inoculum were validated by dilution plating on BG agar plates. At indicated timepoints, nasal cavities, trachea and lungs excised from the mice were homogenized in PBS using a tissue disruptor (BeadMill24, Thermo Scientific). Samples were then serially diluted (10-fold) in PBS, plated on BG agar with streptomycin and incubated at 37°C for 2 days for bacterial enumeration. To obtain serum, blood was collected by cardiac puncture 30 days post-inoculation and centrifuged at 3000 rpm for 20 min, and serum was collected and stored at −20°C until further use.

In vivo complementation

Groups of four mice were inoculated with ~500 cfu of either RB50 or an erythromycin-resistant clone from lineage 1, p6 (EmR1 p6), or a 1:1 mixture of the two (~250 cfu each). At 7 days post-inoculation the ability of the EmR1 p6 strain to colonize the nasal cavity was assessed by plating the nasal cavity homogenates on BG agar plates with and without erythromycin (20 mg/L). Erythromycin concentrations were chosen to differentiate between the WT and p6 resistant mutant colonies.

In vitro experiments

Cytotoxicity

The cytotoxicity of WT and mutant bacteria against mouse RAW 264.7 cells (moi 10:1) was tested using the CytoTox Nonradioactive Cytotoxicity Assay Kit (Promega) following supplied protocols. Cytotoxicity was recorded as the percentage activity of lactate dehydrogenase release compared with that observed from fully lysed cells.
Resistance leads to loss of virulence

Adherence

Adherence of bacteria to human lung epithelial cell line A549 (ATCC) was assessed in 24-well plates at an moi of 10.1 (bacteriaccell). Rates were centrifuged at 300 g for 10 min to synchronize exposure of the cells to bacteria. Following incubation for 5 min at 37°C, the supernatant was aspirated, and the epithelial cells washed (four times) with 1 mL of PBS. Cells were then lysed with 0.1% sodium deoxycholate. Adherent bacteria associated with cells were enumerated by dilution plating on BG agar.

Cytokine assays

RAW 264.7 cells were cultured in RPMI 1640 (Gibco, Life Technologies) supplemented with 10% FBS (Gibco, Life Technologies) and 100 U/mL penicillin/streptomycin (Gibco, Life Technologies) to 90% confluency. Macrophages were washed twice with PBS and fresh medium was added. B. bronchiseptica strain RB50 grown in SS medium to the mid-log phase, as well as the four strains isolated from p1, were added to the cells at an moi of 100. At 4 h post-inoculation, TNF-α and IFN-γ were measured using a DuoSet ELISA system (R&D Systems) following the manufacturer’s protocols.

RT-PCR

Transcript expression of virulent-phase genes (cyaA and pmr) and non-virulent-phase genes (cheZ2 and flhD) was analysed using RNA extracted from mid-log phase cultures grown by shaking at 200 rpm at 37°C in SS medium (virulence-inducing conditions). Expression levels were calculated with the ΔΔCt method using recA for normalization and B. bronchiseptica RB50 as the reference. Data were analysed using DataAssist™ version 3.0 (Applied Biosystems).

Statistical analysis

Statistical analyses of differences between the WT and mutant groups were performed using Student’s t-test of significance, with P < 0.05 considered to be statistically significant.

Results

B. bronchiseptica rapidly develops high levels of antibiotic resistance

To examine if the low reported incidence of erythromycin-resistant B. bronchiseptica was due to an inherently low ability to develop resistance de novo, we exposed erythromycin-susceptible B. bronchiseptica RB50 (WT strain, isolated from a rabbit) to gradually increasing concentrations of erythromycin. We started with four independent cultures to establish four antibiotic-resistant lineages (L1–L4) by serially transferring bacteria that resisted the antibiotic. Standard deviation bars are removed for clarity.

Resistance leads to loss of virulence

The four independent lineages displayed modest variability in their resistance levels, which is likely a reflection of variation in the specific nature of mutations from which their resistances are derived. The resistant clones were all genetically stable, with the mutants retaining their respective resistance thresholds even after several passages without the antibiotic (data not shown). Growth of resistant bacteria on BG blood agar at 37°C revealed that all four lineages of antibiotic-resistant mutants lacked the characteristic haemolytic zone of clearance that normally surrounds WT bacterial colonies. Non-haemolytic colonies are known to arise spontaneously among WT cultures of B. bronchiseptica and p6 isolated from four independent antibiotic-resistant lineages (L1–L4).

Erythromycin-resistant mutants are deficient in colonizing mice

The in vitro haemolysis of blood by B. bronchiseptica is mediated by adenylate cyclase toxin (ACT), one of the several virulence factors of B. bronchiseptica. During primary infections, B. bronchiseptica can elicit an inflammatory response in the lungs of infected mice. This results in the loss of the bacterial population, which may be due to the haemolytic activity of ACT (R. M. Shotton, unpublished observations). Erythromycin-resistant mutants were unable to elicit this inflammatory response (Figure 1). The four independent lineages displayed modest variability in their resistance levels, which is likely a reflection of variation in the specific nature of mutations from which their resistances are derived. The resistant clones were all genetically stable, with the mutants retaining their respective resistance thresholds even after several passages without the antibiotic (data not shown). Growth of resistant bacteria on BG blood agar at 37°C revealed that all four lineages of antibiotic-resistant mutants lacked the characteristic haemolytic zone of clearance that normally surrounds WT bacterial colonies. Non-haemolytic colonies are known to arise spontaneously among WT cultures of B. bronchiseptica at frequencies of ~1/10000 cfu. To assess the contribution of erythromycin exposure to the recovery of non-haemolytic colonies, we grew liquid cultures of WT bacteria in the presence of sublethal amounts of erythromycin (0, 1, 2 or 3 mg/L). After plating the bacteria on three consecutive days of growth at 37°C, we observed a significant dose-dependent increase in numbers of non-haemolytic colonies for 2 and 3 mg/L starting from the second day (Figure 2).

We tested whether the haemolytic phenotype of the passaged erythromycin-resistant mutants would be restored via reversion or compensatory mutations by serially transferring the mutants on plates without antibiotic. Five consecutive passages without antibiotic failed to restore haemolysis for any of the lineages, indicating that the phenotype was stable (data not shown).
factors\textsuperscript{18,19} under the control of a major virulence regulator of \textit{Bordetella}, the BvgAS system.\textsuperscript{20} The BvgAS system senses ambient conditions and induces a virulent (Bvg\textsuperscript{+}), intermediate (Bvg\textsuperscript{i}) or avirulent (Bvg\textsuperscript{−}) phase in the bacteria. Whole-cell antigenic profiles of the antibiotic-resistant mutants from different lineages were similar to each other and to a previously described avirulent \textit{D.bvg} mutant derivative (RB54),\textsuperscript{21} although differences between the latter and the resistant mutants were also evident (Figure S1, available as Supplementary data at JAC Online), raising the question of how these differences would affect colonization and growth in mice. We examined the ability of a resistant clone (EmR1) from the first passage (p1) to infect mice by inoculating groups with 150, 1500 or 15000 cfu by delivery to the external nares in 5 l Lo fP B S . The results showed that WT bacteria successfully colonized mice even when inoculated with as few as 150 cfu, while the mutants were barely detectable even when mice were inoculated with 15000 cfu (100 times more) (Figure 3a). We further compared the colonization efficiencies of randomly chosen clones from all the four lineages of p1 mutants with RB54 in mice inoculated with a high dose of 15000 cfu of each strain. As expected, WT bacteria grew in numbers \textsuperscript{24}10-fold higher than inoculation levels. In contrast, the Bvg\textsuperscript{−} mutant and EmR strains colonized the mice at levels 100- to 1000-fold lower than WT, indicating that the severity of the colonization defect in all four antibiotic-resistant lineages was similar to the severe defect of the Bvg\textsuperscript{−} mutant (Figure 3b). Using growth as an index of general fitness, we tested whether reduced fitness of the mutants could explain the failure to efficiently colonize mice. However, we observed that the resistant mutants grew more vigorously than the WT strain, excluding this possibility (Figure S2).

**Antibiotic-resistant mutants have decreased virulence**

The ability of \textit{B. bronchiseptica} to colonize mice is dependent on its ability to coordinate the activity of many virulence factors to mediate its interaction with the host. We tested the mutants for any alterations to several established phenotypes \textit{in vitro}. We noted differences in two critical phenotypes associated with \textit{in vivo} virulence (Figure 4a and b). First, \textit{in vitro} cytotoxicity of \textit{B. bronchiseptica} directed against mammalian cells is closely associated with its virulence.\textsuperscript{22} We observed a significant decrease (\textit{P} < 0.001) in the cytotoxicity of the mutants compared with the WT when tested against mouse RAW 264.7 macrophage-like cells. Second, the mutants exhibited reduced adherence (\textit{P} < 0.01) towards cultured human lung epithelial cells (A549), adherence being an important phenotype required for establishing colonization of the respiratory tract and immunomodulation.\textsuperscript{23,24} We further noted a significantly reduced ability of the mutants to induce the pro-inflammatory cytokine TNF-\textgreek{a} and modestly reduced amounts of IFN-\gamma from RAW 264.7 cells (Figure 4c). Together, these observations suggest that the antibiotic-resistant mutants have lost their robust virulence phenotype.

Interestingly, although the average MIC range for the resistant strains in the early passages falls within the same range as that for...
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The MIC range of RB50 BvgS, amu tants strat 25 indicating that continued exposure to the antibiotic further increased the MIC values, associated with 150, 1500 or 15000 cfu of B. bronchiseptica. The loss of the ability to colonize mice along with the loss of virulence-associated phenotypes in the mutants closely resembles the avirulent phenotype of B. bronchiseptica in the Bvg– state.26 To evaluate this similarity, we compared EmR mutants with a B. bronchiseptica mutant (strain RB54) in which the bvgS gene is deleted, locking the mutant in the Bvg– state. We examined the expression profiles of the WT and EmR and Bvg– phase-locked bacterial mutants grown at 37°C in liquid medium for genes expressed in the Bvg+ phase [cytA (adenylate cyclase toxin) and prn (pertactin)] and Bvg– phase [cheZ (chemotactin) and flhD (flagella)].27 WT RB50 repressed transcription of cheZ and flhD but induced expression of cytA and prn, whereas the bvgS deletion strain RB54 showed the reverse pattern. All four EmR mutants resembled the bvgS mutant, rather than the WT, with reduced expression of virulence genes and higher expression of motility/chemotaxis genes (Figure 5). These results demonstrate a pattern of altered expression of virulence genes that consistently accompanies increased antibiotic resistance in these four independent lineages, which may explain their defects in mouse infections.

Overall, these results indicate that in the course of antibiotic exposure and development of resistance, B. bronchiseptica attains a phenotype similar, though not identical, to the Bvg– phase.

WT bacteria fail to complement mutants for host colonization

Since many of the known virulence factors, such as ACT, in Bordetella species are secreted,28 WT bacteria could potentially complement such defects, allowing the resistant mutants to colonize and spread among their hosts. To test this hypothesis, we inoculated separate groups of mice with 500 cfu of WT RB50, the EmR1-resistant mutant from p6, or a 1:1 mixed population of the two. At 7 days post-inoculation, we found that WT bacteria had colonized the nasal cavity at the expected levels; however, no erythromycin-resistant bacteria were detected, indicating that the presence of WT bacteria did not facilitate the colonization of the resistant mutant (Figure 6). Bacterial colonization levels in the trachea were ~1000-fold lower than in the nasal cavity, but here too no antibiotic-resistant mutants could be detected. Similarly, the lungs, which had low and inconsistent levels of colonization, showed no resistant mutants. These results indicate that production of factors such as ACT by the WT bacteria did not correct the colonization deficiency of the antibiotic-resistant mutant, suggesting a more complex defect, and indicating that emerging resistant mutants would be unlikely to survive in their host when they arise in response to antibiotic treatment.

Discussion

Although erythromycin has been used extensively in veterinary and medical practice for treating Bordetella spp. infections for decades, the frequency of erythromycin resistance among Bordetella spp. is conspicuously low. Here, we have investigated erythromycin resistance in B. bronchiseptica, a species that, though having close phylogenetic links to the human-restricted B. pertussis, has a broad host range infecting various wild and domesticated animals. In vitro experiments show that B. bronchiseptica readily acquires resistance when exposed to subinhibitory levels of erythromycin. However, the resistant strains that arose were severely deficient in colonizing mice even when inoculated with 15000 cfu, 100-fold above our standard inoculation dose. All four independent lineages

Antibiotic-resistant mutants resemble an engineered bvgS mutant

The loss of the ability to colonize mice along with the loss of virulence-associated phenotypes in the mutants closely resembles
of the mutants that we investigated were similar to the Bvg phase-locked B. bronchiseptica mutant (RB54), which lacks the ability to switch to the virulent Bvg phase required for host colonization. Deficient host colonization would likely impede transmission and spread of antibiotic-resistant bacteria in a population.

The association between acquisition of antibiotic resistance and loss of virulence has been described in other pathogens in vitro and is typically ascribed to a fitness cost. Such costs are believed to be overcome by compensatory mutations that restore fitness, antibiotic susceptibility or virulence. However, we observed no loss of resistance and we did not observe the reversion of the haemolytic phenotype among the B. bronchiseptica mutants even after several passages without antibiotics. In addition, the resistant mutants grew robustly in liquid medium, indicating that no obvious fitness cost accompanied the acquisition of resistance.

Growth in the presence of low concentrations of erythromycin has been previously reported to induce resistance in S. aureus, attaining MIC levels up to 30-fold higher than for uninduced bacteria. However, such high induced resistance is typically associated with horizontally acquired elements and characteristically decreases once the antibiotic is removed. Studies conducted on B. pertussis exposed to erythromycin also reported reversible changes between virulent and avirulent phases, along with concomitant increases in resistance. However, the resistance observed in our study is clonally preserved, retained in the absence of antibiotics and likely associated with genomic changes. Finally, the recent observation that B. bronchiseptica has an alternate and independent life cycle associated with amoebae raises the possibility that infections coming from an environmental source could be having a diluting and buffering effect on the animal-to-animal transmission. Such an effect would affect the rapid spread of antibiotic-resistant B. bronchiseptica arising from animals treated with antibiotics.

The processes by which resistance arises and spreads in some pathogens but not in others remain enigmatic. Although the previously proposed trade-off in fitness may partly explain the lack of resistance in other organisms, our results suggest something else might be going on in Bordetella spp. These organisms have recently been shown to have environmental sources including soil and water. In evolutionary terms, these are the environments in which chemicals similar to antibiotics are likely to be encountered. It may be that Bordetella spp. are relatively well adapted to survive such chemical threats, but that resistance is associated with expression of other factors that promote survival in soil and water but are detrimental during respiratory tract infection. Ectopic expression of Bvg phase genes during infection has previously been shown to be sufficient to disrupt the infectious process. For Bordetella spp., and perhaps other pathogens, the acquisition of antibiotic resistance may not be accompanied by loss of fitness, as measured by in vitro growth, but by an altered gene expression.
Figure 5. Comparison of WT *B. bronchiseptica* RB50 and Bvg− phase-locked and antibiotic-resistant mutants. RT-PCR analysis of genes induced in Bvg− [cheZ (chemotactin) and flhD (flagella)] and Bvg+ [cyaA (adenylate cyclase toxin) and prn (pertactin)] phases of growth in RB50 WT, RB54 (Bvg+ phase locked) and EmR1 p1 antibiotic-resistant mutant growing at 37°C. The bars represent the mean ± SEM (n = 3). ***P < 0.0001 (P values of <0.05 were considered statistically significant).

Figure 6. WT *B. bronchiseptica* RB50 are unable to complement an antibiotic-resistant mutant. The graphs show the number of bacteria recovered from nasal cavities from separate groups of mice 7 days after being inoculated with 500 cfu of RB50 WT, the EmR1 erythromycin-resistant mutant from p6, or a 1:1 mixed population of WT and mutant (250 cfu each). Bacteria recovered from mixed-population inoculations were plated on plates either without (-) or with (+) antibiotic. The dotted lines represent the limit of detection.
pattern associated with surviving antibiotics in their evolutionary context. Thus, antibiotic resistance arises rapidly, but leads consistently to a dead-end lineage that cannot propagate and transmit amongst mammalian hosts.

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