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Sublethal Antibiotic Treatment Leads to Multidrug Resistance via Radical-Induced Mutagenesis

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SUMMARY

Antibiotic resistance arises through mechanisms such as selection of naturally occurring resistant mutants and horizontal gene transfer. Recently, oxidative stress has been implicated as one of the mechanisms whereby bactericidal antibiotics kill bacteria. Here, we show that sublethal levels of bactericidal antibiotics induce mutagenesis, resulting in heterogeneous increases in the minimum inhibitory concentration for a range of antibiotics, irrespective of the drug target. This increase in mutagenesis correlates with an increase in ROS and is prevented by the ROS scavenger thiourea and by anaerobic conditions, indicating that sublethal concentrations of antibiotics induce mutagenesis by stimulating the production of ROS. We demonstrate that these effects can lead to mutant strains that are sensitive to the applied antibiotic but resistant to other antibiotics. This work establishes a radical-based molecular mechanism whereby sublethal levels of antibiotics can lead to multidrug resistance, which has important implications for the widespread use and misuse of antibiotics.

INTRODUCTION

There are a number of mechanisms whereby bacteria can develop antibiotic resistance (Dwyer et al., 2009; Hegreness et al., 2008; Livermore, 2003; McKenzie and Rosenberg, 2001), including horizontal transfer of resistance genes (Davies, 1994), drug-specific selection of naturally occurring resistant variants within a population, and increased mutagenesis in hypermutator strains (Andersson, 2003; Chopra et al., 2003). Quinolones, which are DNA-damaging antibiotics, can stimulate the emergence of drug resistance via SOS-independent recombination (López et al., 2007) and through the induction of RecA-mediated processes, including homologous recombination (Drlica and Zhao, 1997) and SOS-regulated error-prone polymerases (Cirz et al., 2005). β-lactams can also induce the SOS response via RecA (Kohanski et al., 2007) and the DpiAB two-component system (Miller et al., 2004), and these drugs have been shown to induce DinB in an SOS-independent fashion, resulting in increased frameshift mutations (Pérez-Capilla et al., 2005).

Antibiotic treatment can also result in multidrug resistance (Cohen et al., 1989), which has been associated with mutations in multidrug efflux pumps, such as AcrAB (Ma et al., 1993). These drug efflux pumps can be regulated by a number of transcription factors, including the superoxide-responsive SoxRS system (Greenberg et al., 1990). In addition, there is evidence that low-level antibiotic treatment can lead to mutations that cause resistance (Girgis et al., 2009); however, the mechanisms underlying this effect are not well understood.

Bactericidal antibiotics, including β-lactams, quinolones, and aminoglycosides, can stimulate bacteria to produce reactive oxygen species (ROS) (Dwyer et al., 2007; Kohanski et al., 2007, 2008), which are highly deleterious molecules that can interfere with the normal functions of oxygen-respiring organisms (Brumaghim et al., 2003; Fridovich, 1978; Imlay, 2006). Certain ROS, such as hydroxyl radicals, can directly damage DNA and lead to an accumulation of mutations (Demple and Harrison, 1994; Friedberg et al., 2006; Imlay et al., 1988). Oxidative DNA damage also activates the error-prone SOS response (Carlsson and Carpenter, 1980; Imlay and Linn, 1986, 1987) and error-correcting repair systems such as the "GO" repair system (Michaels and Miller, 1992; Miller, 1996). In this study, we hypothesized that ROS formation due to treatment with low levels of bactericidal antibiotics leads to an increase in mutation rates, which can result in the emergence of multidrug resistance. We thus consider a possible molecular mechanism whereby bactericidal antibiotics act as active, reactive mutagens.

RESULTS

To test the above hypothesis, we examined mutation rates in *E. coli* strain MG1655 following treatment with low levels of the bactericidal antibiotics norfloxacin (quinolone), ampicillin (β-lactam), and kanamycin (aminoglycoside), respectively. Mutation

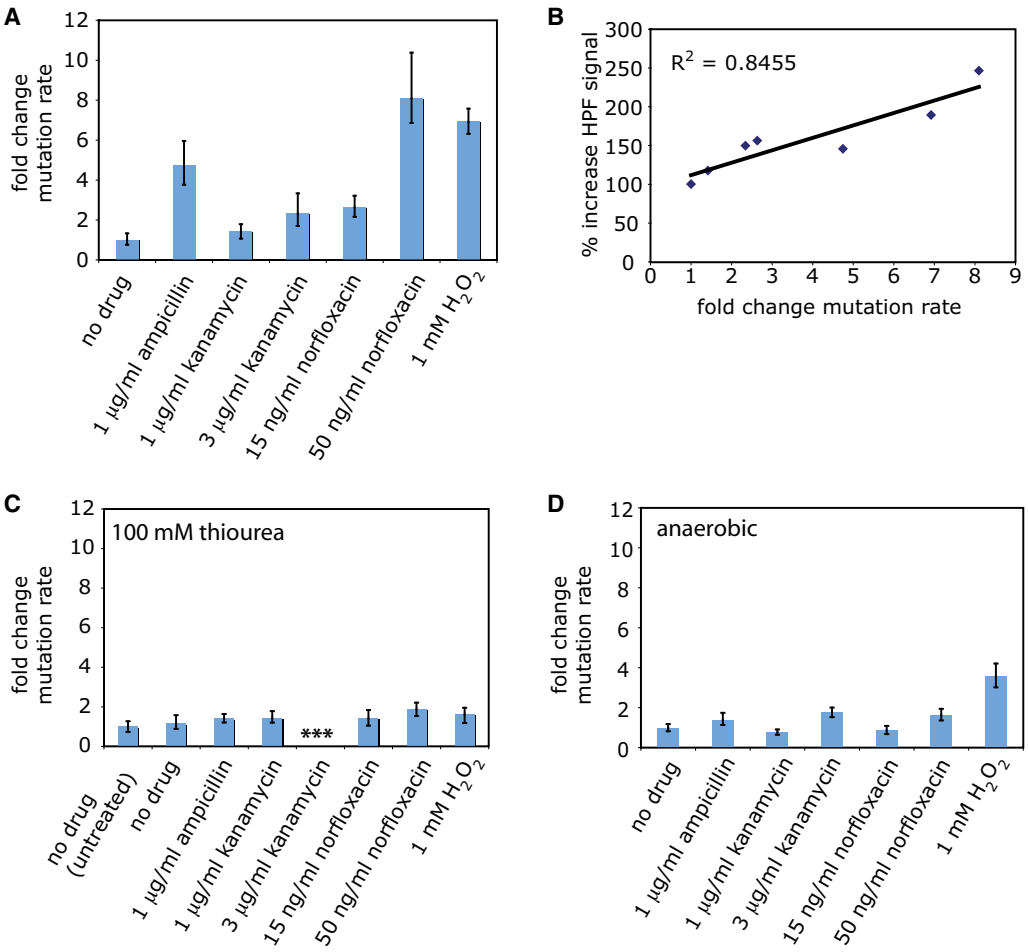

Figure 1. Low Levels of Bactericidal Antibiotics Increase Mutation Rate Due to Reactive Oxygen Species Formation

(A) Fold change in mutation rate (mean ±95% confidence interval [CI]) relative to an untreated control (no drug) for wild-type *E. coli* (MG1655) following an overnight treatment with 1 μg/ml ampicillin, 1 μg/ml kanamycin, 3 μg/ml kanamycin, 15 ng/ml norfloxacin, 50 ng/ml norfloxacin, or 1 mM hydrogen peroxide (H_2O_2).
(B) Correlation between oxidative stress levels (HPF fluorescence) and fold change in mutation rate for wild-type *E. coli* for the treatments described in (A).
(C and D) Fold change in mutation rate (mean ±95% CI) relative to an untreated control (no drug) for wild-type *E. coli* following an overnight treatment with 100 mM thiourea and no drug, 1 μg/ml ampicillin, 1 μg/ml kanamycin, 3 μg/ml kanamycin, 15 ng/ml norfloxacin, 50 ng/ml norfloxacin, or 1 mM hydrogen peroxide (H_2O_2) under aerobic growth conditions with 100 mM thiourea (C) or anaerobic growth conditions (D). See also Figure S2.

rates were determined by plating aliquots of treated cultures onto rifampicin plates, counting rifampicin-resistant colonies, and using the MSS maximum likelihood method (Rosche and Foster, 2000) to estimate the number of mutation events per culture (see Experimental Procedures for additional details). The mutation rate for untreated wild-type *E. coli* was approximately 1.5×10^{-8} mutations/cell/generation.

Treatment with 1 μg/ml ampicillin, 3 μg/ml kanamycin, 15 ng/ml norfloxacin, or 50 ng/ml norfloxacin resulted in significant increases in the mutation rate relative to an untreated control (Figure 1A). Treatment with 1 μg/ml kanamycin resulted in a modest increase in mutation rate (Figure 1A). The largest increases in mutation rate were seen following treatment with ampicillin or 50 ng/ml norfloxacin (Figure 1A). These changes were on par with the increase in mutation rate observed following treatment with 1 mM hydrogen peroxide (Figure 1A), a concentration of hydrogen peroxide known to induce hydroxyl radical formation via Fenton chemistry (Imlay et al., 1988). To determine if there is a correlation between these changes in mutation rate and ROS formation, we examined radical levels using the radical-sensitive dye 3′-(p-hydroxyphenyl) fluorescein (HPF) (Setsukinai et al., 2003) (see Experimental Procedures for more details). We found a significant correlation ($R^2 = 0.8455$) between the fold change in mutation rate and peak HPF signal for the treatments described above (Figure 1B).

The strong correlation between ROS formation and fold change in mutation rate following treatment with bactericidal antibiotics suggests that ROS actively contribute to bactericidal drug-induced mutagenesis. To test if this is indeed the case, we added 100 mM thiourea to wild-type *E. coli* treated with antibiotics or hydrogen peroxide at the concentrations noted above (Figure 1C). Thiourea is a potent hydroxyl radical scavenger that mitigates the effects of hydroxyl radical damage in both prokaryotes and eukaryotes (Novogrodsky et al., 1982; Repine

et al., 1981; Touati et al., 1995). We have previously shown that thiourea reduces hydroxyl radical formation and cell killing following treatment with bactericidal antibiotics (Kohanski et al., 2007).

The addition of thiourea significantly reduced the mutation rate to near untreated levels following the addition of 1 mM hydrogen peroxide, norfloxacin, or ampicillin (Figure 1C). Interestingly, we were unable to detect any rifampicin-resistant colonies after plating up to 10^9 cells following treatment with both 3 μg/ml kanamycin and thiourea (Figure 1C). However, we were able to detect rifampicin-resistant colonies after scaling up the system to 1 L flasks and plating up to 10^{10} cells following treatment with both 3 μg/ml kanamycin and thiourea (data not shown). These results suggest a role for kanamycin-mediated interference with ribosome function and translation, in the absence of oxidative stress, on significantly lowering mutation rate.

To further demonstrate that antibiotic-mediated ROS formation has a mutagenic component, we examined mutation rates under anaerobic growth conditions (see Experimental Procedures for additional details) following treatment of wild-type *E. coli* with antibiotics or hydrogen peroxide as described above (Figure 1D). We observed mutation rates near untreated levels for all antibiotic treatments tested (Figure 1D). Treatment with 1 mM hydrogen peroxide, which results in direct addition of an oxidant, led to an increase in mutation rate relative to the no-drug control under anaerobic growth conditions (Figure 1D), but this increase was considerably smaller than that exhibited under aerobic growth conditions (Figure 1A).

Antibiotic-resistant strains can arise via drug-mediated selection of preexisting antibiotic-resistant variants that occur naturally within a population (Livermore, 2003). Antibiotic-induced oxidative stress may be an additional mechanism that allows for the accumulation of mutations that increase resistance to drugs, irrespective of the drug target of the applied antibiotic. To test this, we measured changes in the minimum inhibitory concentration (MIC) of wild-type *E. coli* over a period of 5 days of selective growth for the following antibiotics: norfloxacin, kanamycin, ampicillin, tetracycline, and chloramphenicol. During the growth period, the cultures were exposed to no drug, norfloxacin, ampicillin, or kanamycin (see Experimental Procedures for more details). In all cases, growth in the absence of antibiotics did not change the MIC for any of the drugs tested (data not shown).

Treatment with 25 ng/ml norfloxacin led to an increase in the MIC for norfloxacin and kanamycin (Figure S1A). The observed increases in MIC following treatment with norfloxacin were concentration dependent (see Supplemental Information for more details). Treatment of wild-type *E. coli* with 3 μg/ml kanamycin led to an increase in the MIC for kanamycin and minimal increases in the MIC for norfloxacin and ampicillin, respectively (Figure S1C). The MIC for tetracycline and chloramphenicol did not change (Figure S1C), indicating that kanamycin treatment may not lead to mutants resistant to other classes of ribosome inhibitors.

Treatment of wild-type *E. coli* with 1 μg/ml ampicillin for 5 days led to an increase in the MIC to different levels for ampicillin, norfloxacin, kanamycin, tetracycline, and chloramphenicol (Figure 2A). These results show that treatment with a β-lactam can

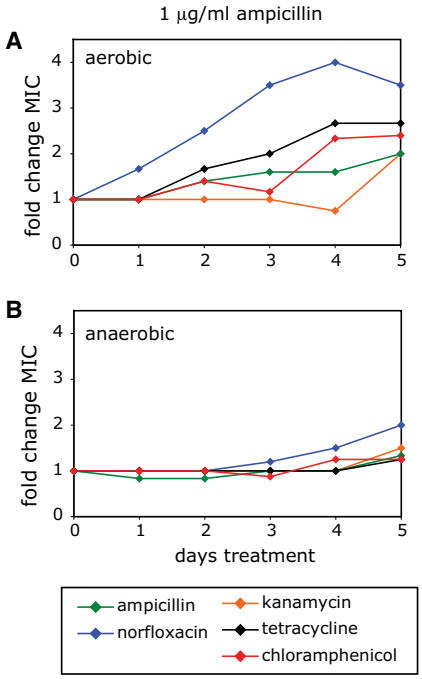

Figure 2. Low Levels of Bactericidal Antibiotics Can Lead to Broad-Spectrum Increases in MIC Due to ROS-Mediated Mutagenesis

(A and B) Fold change in MIC relative to an aerobic no-drug control for ampicillin, norfloxacin, kanamycin, tetracycline, and chloramphenicol, following 5 days of growth in the presence of 1 μg/ml ampicillin under aerobic (A) or anaerobic (B) growth conditions. See also Figure S1.

stimulate formation of mutants that are potentially resistant to a wide range of antibiotics. Cultures that had been grown for 5 days in the presence of low levels of ampicillin were shifted to a drug-free environment and grown without any ampicillin for 2 additional days. The MICs, which were increased after 5 days of ampicillin treatment (Figure 2A), remained elevated and did not change significantly following 2 days of growth in the absence of ampicillin (Figure S1D). These findings demonstrate that the observed increases in MIC are stable and not due to a transient adaptation to growth in the presence of ampicillin.

To determine if the observed increases in MIC were related to antibiotic-mediated ROS formation, we measured the MIC for ampicillin, norfloxacin, kanamycin, tetracycline, and chloramphenicol, respectively, following treatment with no drug or 1 μg/ml ampicillin under anaerobic growth conditions. Untreated anaerobic growth had no effect on MIC relative to untreated aerobic growth (data not shown). Following treatment with 1 μg/ml ampicillin under anaerobic conditions, we observed almost no increase in MIC for ampicillin, kanamycin, tetracycline, or chloramphenicol (Figure 2B). The MIC for norfloxacin exhibited a small increase by day 5 (Figure 2B); however, this change in MIC was much smaller than the increase in MIC for norfloxacin following ampicillin treatment under aerobic growth conditions (Figure 2A). These results suggest that ROS formation due to treatment with low levels of bactericidal antibiotics can lead to mutagenesis and the emergence of bacteria resistant to a wide range of antibiotics.

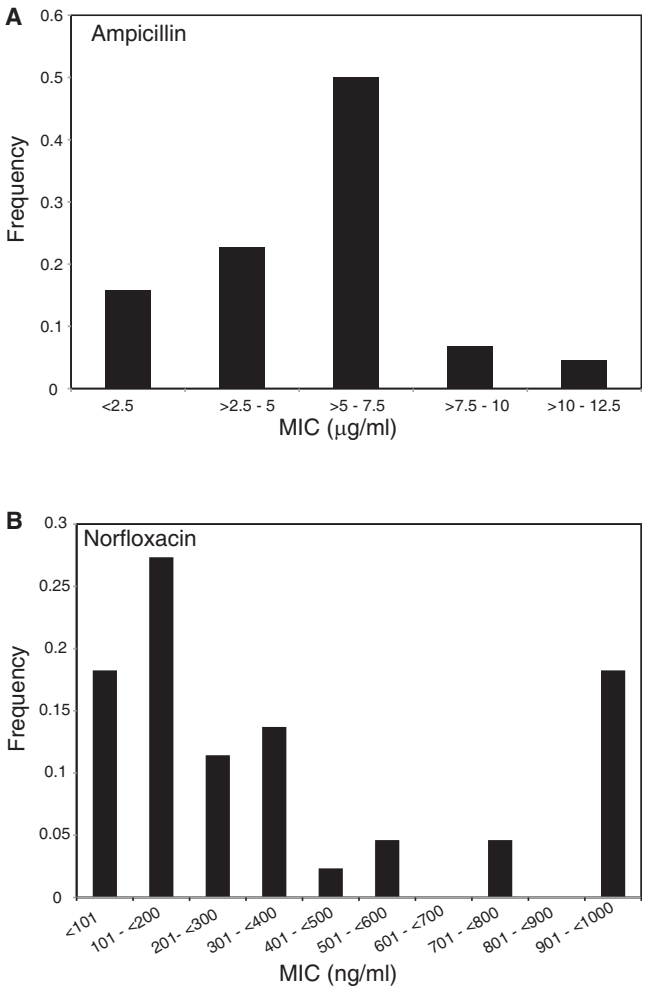

Figure 3. Ampicillin Treatment of *E. coli* Results in Heterogeneous Increases in MIC for Ampicillin and Norfloxacin

(A and B) Shown are the distributions of ampicillin (A) or norfloxacin (B) MICs for 44 ampicillin-treated isolates. The maximum growth-inhibitory concentration tested for norfloxacin was 1000 ng/ml, and the MICs for these isolates may be ≥1000 ng/ml.

Drug resistance may not always be uniform throughout a population. Some cells within a population may remain susceptible to the antibiotic, whereas other cells display varying degrees of drug resistance (de Lencastre et al., 1993), a phenomenon referred to as heteroresistance. Antibiotic-stimulated, ROS-mediated mutagenesis could be a mechanism that stimulates the formation of a range of mutations that result in varying MICs within a population of cells. We sought to determine if the observed increases in population-level MIC for ampicillin following 5 days of treatment with 1 μg/ml ampicillin (Figure 2A) exhibited heterogeneity in MIC at the single-colony level.

We isolated individual colonies following ampicillin treatment and measured the MIC of each clone to ampicillin. We found that these isolates exhibited a range of resistance to ampicillin (>2.5–12.5 μg/ml), with some isolates remaining completely susceptible (≤2.5 μg/ml) to treatment with this drug (Figure 3A). We also found that the MICs for these isolates to norfloxacin ranged from <100 ng/ml (completely susceptible) to ≥1000 ng/ml (Figure 3B). Although levels of resistance from clinical isolates are typically quite high (with MICs in the range of 10–1000 μg/ml for norfloxacin [Becnel Boyd et al., 2009]), the upper ranges of the MICs for ampicillin or norfloxacin observed here (Figure 3) are near the peak serum concentrations for these drugs (Bryskier, 2005), indicating that these MICs might be near the limit for the amount of drug a human can tolerate. These data show that heterogeneous increases in MIC to ampicillin arise in *E. coli* following treatment with low levels of ampicillin, and treatment with one drug class can lead to heterogeneous increases in MIC against other classes of antibiotics.

Resistance to multiple antibiotics has been linked to mutations in drug-efflux systems such as the AcrAB multidrug (MDR) efflux pump (George and Levy, 1983; Ma et al., 1993) as well as mutations in transcription factors controlling these systems, such as MarA (Alekshun and Levy, 1997), Rob (Ariza et al., 1995), and SoxS (Greenberg et al., 1990). Our results suggest that ROS-mediated DNA damage induced by low levels of bactericidal antibiotics can result in mutations in a wider range of genes, potentially in some unrelated to the applied antibiotic and drug efflux systems. This implies that treatment with ampicillin, for example, may generate mutants that are not ampicillin resistant but are resistant to other antibiotics.

To determine if these types of resistant strains arise, we examined multidrug resistance following 5 days of treatment with 1 μg/ml ampicillin or no treatment. Mutants from ampicillin-treated or untreated cultures were selected on plates containing norfloxacin, ampicillin, kanamycin, tetracycline, and chloramphenicol, respectively. From this primary selection, we determined cross-resistance to the other four antibiotics via replica plating (see Experimental Procedures for additional details). We found substantially more primary resistant colonies and higher rates of cross-resistance following ampicillin treatment as compared to no treatment (Table 1). Ampicillin-selected mutants displayed a range of cross-resistance to the other classes of antibiotics and showed a strong correlation (89% cross-resistance) with norfloxacin resistance (Table 1). We also found that ampicillin-treated cells selected originally on the basis of norfloxacin or kanamycin resistance were only 75% and 63% cross-resistant to ampicillin, respectively (Table 1). Interestingly, primary resistance selection with the static drugs tetracycline and chloramphenicol yielded isolates that were always (100%) cross-resistant to ampicillin (Table 1); this effect deserves further study. Also of note, ampicillin-treated, kanamycin-resistant strains were found to have very low cross-resistance to tetracycline (7%) and no cross-resistance with chloramphenicol (Table 1). This is consistent with previous work showing a lack of cross-resistance to tetracycline or chloramphenicol following selective treatment with aminoglycosides (Grassi, 1979). While the majority of these multidrug cross-resistant strains exhibit resistance against the treatment drug, ampicillin, our results demonstrate that treatment with ampicillin can also generate mutants that are not resistant to ampicillin yet are resistant to other classes of antibiotics.

We sought to determine if some of the ampicillin-treated, cross-resistant isolates had acquired mutations in specific

Table 1. Cross-Resistance Following Ampicillin Treatment and Primary Resistance Selection with Five Different Classes of Antibiotics

E. coli Control Strain	Percent Cross-Resistant Following Ampicillin Treatment				
	Norfloxacin	Ampicillin	Kanamycin	Tetracycline	Chloramphenicol
Primary Selection					
Norfloxacin	100% (40/40)	75% (30/40)	25% (10/40)	23% (9/40)	23% (9/40)
Ampicillin	89% (77/87)	100% (87/87)	20% (17/87)	54% (47/87)	21% (18/87)
Kanamycin	20% (17/83)	63% (52/83)	100% (83/83)	7% (6/83)	0% (0/83)
Tetracycline	79% (63/80)	100% (80/80)	14% (11/80)	100% (80/80)	78% (62/80)
Chloramphenicol	87% (67/77)	100% (77/77)	35% (27/77)	100% (77/77)	100% (77/77)
	Percent Cross-Resistant Following No-Drug Treatment				
	Norfloxacin	Ampicillin	Kanamycin	Tetracycline	Chloramphenicol
Primary Selection					
Norfloxacin	100% (10/10)	0% (0/10)	0% (0/10)	0% (0/10)	0% (0/10)
Ampicillin	0% (0/10)	100% (10/10)	0% (0/10)	0% (0/10)	0% (0/10)
Kanamycin	0% (0/15)	0% (0/15)	100% (15/15)	0% (0/15)	0% (0/15)
Tetracycline	100% (1/1)	0% (0/1)	0% (0/1)	100% (1/1)	0% (0/1)
Chloramphenicol	100% (1/1)	0% (0/1)	0% (0/1)	0% (0/1)	100% (1/1)

Wild-type *E. coli* were treated with 1 mg/ml ampicillin or no drug for 5 days. These ampicillin-treated or untreated cells were spread on plates containing norfloxacin, ampicillin, kanamycin, tetracycline, or chloramphenicol, and mutants resistant to the individual drugs were isolated. Resistance to the other four classes of antibiotics was determined by replica plating of the primary-selected strains onto plates containing the respective antibiotic. Shown is percent resistance (resistant colonies/total primary resistant colonies). Note: Double the volume of no-drug control cells were plated for primary resistance selection for *E. coli* as compared to the ampicillin-treated cells.

antibiotic targets or in genes making up the common oxidative damage cell death pathway induced by bactericidal antibiotics (Kohanski et al., 2007, 2008), or if the observed cross-resistance (Table 1) was solely a function of altered drug efflux. We examined six norfloxacin-resistant isolates, six kanamycin-resistant isolates, and the untreated control strain. We sequenced the following genes where mutations could potentially lead to an increase in drug resistance: *gyrA*, *gyrB*, *rpsL*, *ampC*, *icdA*, *arcA*, *cpxA*, *sdhB*, *iscR*, *tolC*, *marRA* and its promoter region, and *acrA* and its promoter region. *gyrA* and *gyrB* code for the subunits of DNA Gyrase; the known target of quinolones, *rpsL*, encodes a component of the 30S subunit of the ribosome and has been associated with aminoglycoside resistance; *ampC* has been associated with ampicillin resistance; *icdA*, *arcA*, *cpxA*, *sdhB*, and *iscR* are genes involved in the common mechanism of cell death; and *tolC*, *marRA* and its promoter region, and *acrA* and its promoter region are involved in multidrug efflux.

We found that 3 of the 6 norfloxacin-resistant isolates contained point mutations in *gyrA* that resulted in a substitution of glycine for aspartic acid at amino acid 82 in one isolate and a substitution of tyrosine for aspartic acid at amino acid 87 in two other isolates (Figure 4A). We also found that 1 of these 6 norfloxacin-resistant isolates, which did not have a mutation in *gyrA*, had a point mutation resulting in the conversion of serine to phenylalanine at residue 464 of GyrB (Figure 4B). Interestingly, the point mutations we found in *gyrA* and *gyrB* are all in the quinolone resistance-determining regions of GyrA and GyrB, respectively, and these mutations have been observed in clinical isolates of *Bacteroides fragilis* (Oh et al., 2001), *Salmonella enterica* (Weill et al., 2006), and *Pseudomonas aeruginosa* (Mouneimné et al., 1999).

As noted above, mutations in *rpsL* have been associated with aminoglycoside resistance. We found that 2 of the 6 kanamycin-resistant isolates had point mutations in *rpsL*. These mutations led to a frameshift and truncated form of RpsL in both isolates (Figure 4D). It is possible that these mutations contribute to kanamycin resistance in these isolates.

Among the ampicillin-treated, drug-resistant mutants, we did not find any mutations in *ampC* (data not shown), a gene associated with ampicillin resistance. We also did not find any mutations in *icdA*, *cpxA*, *sdhB*, or *iscR* (data not shown). Interestingly, we did find a single insertion mutation in *arcA* in one of the drug-resistant isolates. ArcA is a two-component system transcription factor containing a sensor domain and a DNA-binding domain, and the mutation we found results in a truncated ArcA protein that is missing the DNA-binding element of the protein (Figure 4E). We have previously shown that two-component systems are important elements in the common mechanism of cell death, and a knockout of *arcA* is more tolerant to treatment with ampicillin and kanamycin compared to norfloxacin (Kohanski et al., 2008). This isolate is resistant to ampicillin and kanamycin, but not to norfloxacin. This result suggests that mutations leading to low-level antibiotic resistance can occur in genes that are involved in the common mechanism of cell death.

We did not find any mutations in *tolC*, *marRA*, the *marRA* promoter, or *acrA* (data not shown); however, we did find a T-to-A conversion in the promoter upstream of *acrA* (Figure 4C) in one of the norfloxacin-resistant isolates that also had a mutation in *gyrA* (Figure 4A). This promoter mutation occurs within the annotated −35 site of the promoter and the binding site for the repressor transcription factors AcrR and EnvR (Keseler et al., 2005; Miller et al., 2002). The observed mutations could reduce the ability of these repressors to bind to the *acrAB* promoter,

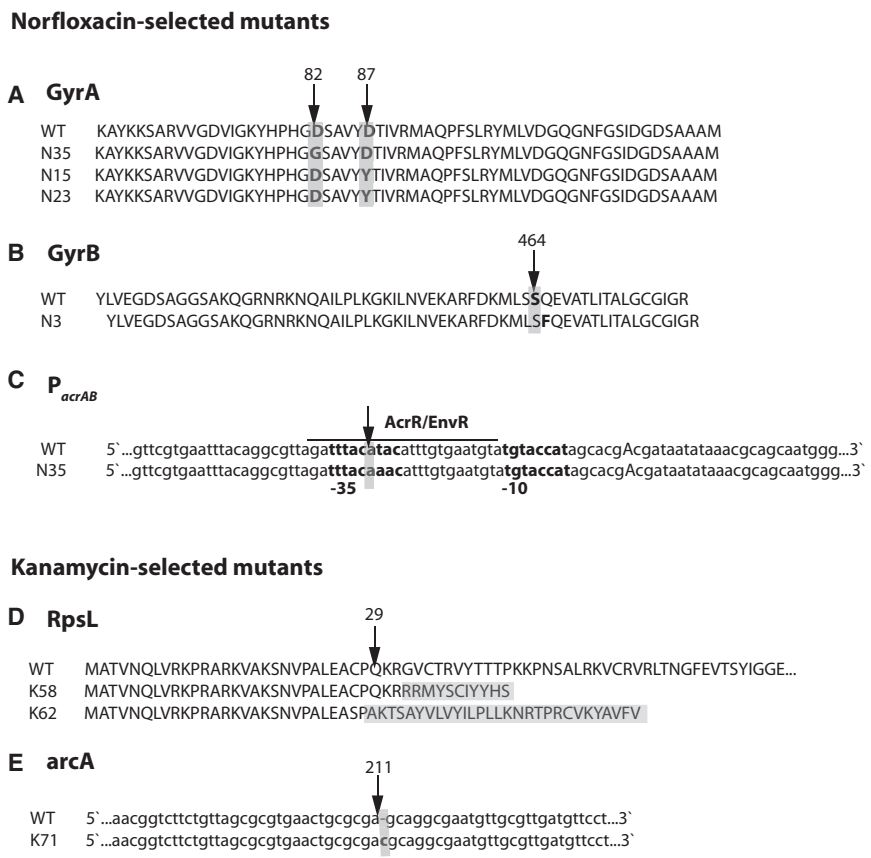

Figure 4. Ampicillin Treatment Leads to the Formation of Norfloxacin-Resistant Isolates with Mutations in *gyrA*, *gyrB*, or the *acrAB* Promoter (P_{acrAB}) and Kanamycin-Resistant Isolates with Mutations in *rpsL* or *arcA*

(A and B) Isolates with point mutation resulting in a D82G or D87Y substitution in GyrA (A) or a S464F substitution in GyrB (B).

(C) T-to-A DNA base pair mutation in the AcrR/EnvR binding site of the −35 region of P_{acrAB}. P_{acrAB} is partially annotated to show the −10 and −35 regions (bold), the transcription start site (capitalized A), and the AcrR/EnvR binding site (underlined).

(D) Isolates with insertion between base pair 92 and 93 (K58) and between base pair 78 and 79 (K62) resulting in truncation of RpsL.

(E) Isolate with a single base pair insertion between base pair 211 and 212 resulting in a truncated ArcA protein missing the majority of the helix-turn-helix (HTH) DNA binding domain. See also Table S1.

which would result in increased pump expression and drug resistance. These sequencing results demonstrate that ampicillin treatment can lead to the formation of norfloxacin-resistant strains with mutations in DNA Gyrase and/or mutations that can affect drug efflux pump activity, which likely contribute to the emergence of multidrug resistance.

To demonstrate that sublethal levels of bactericidal antibiotics can lead to an increase in multidrug cross-resistance in Gram-positive as well as Gram-negative bacteria, we also examined multidrug cross-resistance in *Staphylococcus aureus* following treatment with low levels of ampicillin (35 ng/ml) for 5 days. Previously, we demonstrated antibiotic-mediated ROS formation in *S. aureus* (Kohanski et al., 2007). In the present study, we found substantially more primary resistant *S. aureus* colonies and higher rates of cross-resistance following ampicillin treatment as compared to no treatment (Table 2). Interestingly, we were unable to enrich for tetracycline- or chloramphenicol-resistant *S. aureus* isolates following treatment with low-level ampicillin as compared with the no-drug treatment. This may be due to the lower level of ROS formation we have observed with *S. aureus* (Kohanski et al., 2007).

To demonstrate that these effects are not limited to lab strains, we considered a clinical isolate of *E. coli* from a patient with diarrhea (NCDC C771). We examined multidrug cross-resistance in the clinical isolate following treatment with 1 μg/ml ampicillin (see Experimental Procedures for more details). As with the wild-type strains, we found substantially more primary resistant colonies and higher rates of cross-resistance in the clinical isolates following ampicillin treatment as compared to no treatment. We also found that ampicillin-treated cells selected originally on the basis of norfloxacin or kanamycin resistance were only 11.5% and 21.5% cross-resistant to ampicillin, respectively (Table 3). This further affirms that treatment with ampicillin can generate mutants that are not resistant to ampicillin yet are resistant to other classes of antibiotics.

DISCUSSION

Here, we establish a radical-based molecular mechanism whereby sublethal levels of antibiotics can lead to multidrug resistance. This occurs via bactericidal antibiotic-mediated radical formation that results in the formation of mutations, some of which confer antibiotic resistance. Low-level resistance likely provides a first step toward clinically significant resistance (Goldstein, 2007), and the mechanism we propose and validate here establishes an antibiotic-stimulated mutagenic effect that likely works in conjunction with SOS-induced mutagenesis in the emergence of mutations that confer drug resistance.

Clinical situations where bacteria are exposed to low levels of antibiotics can occur with incomplete treatment of an infection, noncompliance with antibiotic treatment (e.g., a missed pill), and reduced or limited drug accessibility to certain tissues (e.g., bone or cerebrospinal fluid [Bryskier, 2005]). It is possible that mutations arising via antibiotic-mediated oxidative stress could be maintained in the normal bacterial flora of the body and transferred to virulent bacteria via horizontal gene transfer, a mechanism that can be induced by DNA damage (Beaber et al., 2004). Novel therapeutics targeting ROS-forming systems or error-prone DNA damage repair systems may help reduce and contain the spread of new antibiotic-resistant bacteria.

Table 2. Cross-Resistance for *S. aureus* Following Ampicillin Treatment and Primary Resistance Selection with Five Different Classes of Antibiotics

S. aureus	Percent Cross-Resistant Following Ampicillin Treatment				
	Norfloxacin	Ampicillin	Kanamycin	Tetracycline	Chloramphenicol
Primary Selection					
Norfloxacin	100% (59/59)	64% (38/59)	56% (33/59)	36% (21/59)	19% (11/59)
Ampicillin	41% (29/71)	100% (71/71)	18% (13/71)	25% (18/71)	14% (10/71)
Kanamycin	13% (9/68)	60% (41/68)	100% (68/68)	18% (12/68)	15% (10/68)
Tetracycline	0% (0/2)	100% (2/2)	0% (0/2)	100% (2/2)	0% (0/2)
Chloramphenicol	0/0	0/0	0/0	0/0	0/0
	Percent Cross-Resistant Following No-Drug Treatment				
	Norfloxacin	Ampicillin	Kanamycin	Tetracycline	Chloramphenicol
Primary Selection					
Norfloxacin	100% (19/19)	5% (1/19)	26% (5/19)	0% (0/19)	5% (1/19)
Ampicillin	0% (0/13)	100% (13/13)	0% (0/13)	8% (1/13)	0% (0/13)
Kanamycin	2.6% (1/38)	2.6% (1/38)	100% (38/38)	0% (0/38)	8% (3/38)
Tetracycline	0/0	0/0	0/0	0/0	0/0
Chloramphenicol	0/0	0/0	0/0	0/0	0/0

Wild-type *S. aureus* were treated with 35 ng/ml ampicillin or no drug for 5 days. These ampicillin-treated or untreated cells were spread on plates containing norfloxacin, ampicillin, kanamycin, tetracycline, or chloramphenicol, and mutants resistant to the individual drugs were isolated. Resistance to the other four classes of antibiotics was determined by replica plating of the primary selected strains onto plates containing the respective antibiotic. Shown is percent resistance (resistant colonies/total primary resistant colonies).

EXPERIMENTAL PROCEDURES

Strains, Media, and Antibiotics

All experiments were performed with wild-type *E. coli* strain MG1655 (ATCC 700926) in Luria-Bertani (LB) medium (Fisher Scientific; Waltham, MA). For all treatment conditions, we used 1 mM hydrogen peroxide (VWR; West Chester, PA) and the following bactericidal antibiotics: norfloxacin (Sigma; St. Louis), ampicillin, and kanamycin (Fisher Scientific). Bactericidal antibiotics were used at concentrations of 15 ng/ml norfloxacin, 50 ng/ml norfloxacin, 1 μg/ml ampicillin, 1 μg/ml kanamycin, or 3 μg/ml kanamycin. Tetracycline (MP Biomedical; Solon, OH) and chloramphenicol (Fluka; St. Louis) were used for MIC assays, rifampicin (Sigma) for determination of antibiotic resistant rates, and thiourea (Fluka) for radical-quenching experiments. Anaerobic media was made by heating LB in 17 ml Bellco glass hungate tubes (Fisher Scientific) under anaerobic conditions in a Coy anaerobic chamber (Coy Laboratory Products Inc.; Grass Lake, MI) to drive out dissolved oxygen (Norris and Ribbons, 1969). Resazurin (10 mM) (Sigma), which turns clear in the absence of oxygen, was used as an indicator for anaerobic conditions. Multidrug

Table 3. Cross-Resistance for *E. coli* Clinical Isolate NCDC C771 Following Ampicillin Treatment and Primary Resistance Selection with Four Different Classes of Antibiotics

E. coli Clinical Isolate	Percent Cross-Resistant Following Ampicillin Treatment			
	Norfloxacin	Ampicillin	Kanamycin	Chloramphenicol
Primary Selection				
Norfloxacin	100% (78/78)	11.5% (9/78)	1.3% (1/78)	10.3% (8/78)
Ampicillin	13.2% (5/38)	100% (38/38)	2.6% (1/38)	23.9% (9/38)
Kanamycin	15.2% (12/79)	21.5% (17/79)	100% (79/79)	7.6% (6/79)
Chloramphenicol	41.4% (29/70)	45.7% (32/70)	22.9% (16/70)	100% (70/70)
	Percent Cross-Resistant Following No-Drug Treatment			
	Norfloxacin	Ampicillin	Kanamycin	Chloramphenicol
Primary Selection				
Norfloxacin	0/0	0/0	0/0	0/0
Ampicillin	0/0	0/0	0/0	0/0
Kanamycin	2.8% (1/36)	11.1% (4/36)	100% (36/36)	2.8% (1/36)
Chloramphenicol	0/3	0/3	0/3	100% (3/3)

E. coli strain NCDC C771 was treated with 1 μg/ml ampicillin or no drug for 5 days. These ampicillin-treated or untreated cells were spread on plates containing norfloxacin, ampicillin, kanamycin, or chloramphenicol, and mutants resistant to the individual drugs were isolated. Resistance to the other three classes of antibiotics was determined by replica plating of the primary-selected strains onto plates containing the respective antibiotic. Shown is percent resistance (resistant colonies/total primary resistant colonies). Tetracycline cross-resistance was not quantified for NCDC C771, as this strain is resistant to tetracycline (MIC > 35 μg/ml).

resistance was also determined in wild-type *S. aureus* (ATCC 25923) and the *E. coli* clinical isolate NCDC C771 (ATCC 23985).

Determination of Mutation Rate

Mutation rates were examined following 24 hr of growth in the presence of a bactericidal antibiotic. Drug levels were chosen such that there was an observable effect on growth or survival within the first 6 hr after drug addition (Figure S2), followed by "recovery" of the culture to near untreated colony density 24 hr after treatment. All treatment conditions exhibited recovery to near untreated colony density levels, with the exception of 50 ng/ml norfloxacin. This allowed us to compare mutation frequencies for cultures of similar densities following treatment with an antibiotic.

Mutation rates were determined using a rifampicin-based selection method (Giraud et al., 2001). Briefly, an overnight culture of *E. coli* was diluted 1:10,000 into 50 ml LB in a 250 ml baffled flask and grown for 3.5 hr at 37°C and 300 rpm. Cultures were grown at high shaking speeds and in baffled flasks to maximize aeration and ROS formation. The culture was diluted 1:3 into fresh LB containing no drug, an antibiotic, or hydrogen peroxide at the concentrations described above. For experiments with thiourea, thiourea in solid form was added to each diluted culture for a final concentration of 100 mM. Aliquots (1 ml; ten replicates) of these diluted cultures were grown in 14 ml tubes for 24 hr at 37°C and 300 rpm. Aliquots of each treatment were serially diluted and plated on LB-agar plates for colony forming unit per milliliter (cfu/ml) determination. Aliquots of each treatment were also plated on LB-agar plates containing 100 μg/ml rifampicin and grown for 48 hr at 37°C. Colonies were counted at 24 and 48 hr, and the colony count from the 48 hr time point was used to estimate mutation rates. For experiments in anaerobic conditions, cells were diluted 1:10,000 into 15 ml anaerobic LB in sealed hungate tubes to minimize exposure to oxygen. Antibiotic treatments, growth temperature, shaking speed, and sample collection were as described above for the aerobic growth conditions.

The colony counts from the ten replicates were then used in the MSS maximum-likelihood method (Rosche and Foster, 2000; Sarkar et al., 1992) to estimate the number of mutational events per culture. The MSS maximum likelihood method is a recursive algorithm based on the Lea-Coulson function for solving the Luria-Delbruck distribution for a given number of mutational events (Sarkar et al., 1992); its utility has been demonstrated in vitro (Rosche and Foster, 2000). The mutation rate was determined by dividing the number of mutational events per culture by the total number of bacteria plated on the rifampicin plates (Rosche and Foster, 2000). Fold change in mutation rate was determined for all treatments and conditions relative to an untreated MG1655 control. Three biological replicates were run for each treatment condition, and the averages are shown in Figure 1.

ROS Detection Using HPF

To detect ROS formation, we used the fluorescent reporter dye HPF (Invitrogen; Carlsbad, CA) and flow cytometry as previously described (Kohanski et al., 2007). Average fluorescence was determined at 0 (baseline), 1, 3, and 6 hr (normalized to a no-dye control) following antibiotic treatment at the concentrations described above, and peak fluorescence levels were used to determine the change in mean fluorescence relative to baseline (Figure 1B).

Determination of MIC

For wild-type *E. coli*, MICs for norfloxacin, ampicillin, kanamycin, tetracycline, and chloramphenicol were measured over 5 days of treatment with no drug, 25 ng/ml norfloxacin, 50 ng/ml norfloxacin, 1 μg/ml ampicillin, or 3 μg/ml kanamycin. Briefly, an overnight culture of *E. coli* was diluted 1:10,000 into 50 ml LB in a 250 ml baffled flask and grown for 3.5 hr at 37°C and 300 rpm. The culture was diluted 1:3 into fresh LB containing no drug or antibiotics at the above concentrations. Aliquots (1 ml) of these diluted cultures were grown in 14 ml tubes for 24 hr at 37°C and 300 rpm. Each day thereafter for 5 days, in order to avoid mutations arising due to evolution during stationary phase (GASP mutants) (Zinser and Kolter, 2004), cells were diluted 1:1000 into 1 ml LB in a 14 ml tube containing the respective antibiotic and grown for 24 hr at 37°C and 300 rpm.

MICs were also measured for anaerobically grown *E. coli* over 5 days of treatment with no drug or 1 μg/ml ampicillin. Briefly, an overnight culture of *E. coli* was diluted 1:1000 into 15 ml anaerobic LB in sealed hungate tubes containing no drug or 1 μg/ml ampicillin. These cultures were grown in the sealed hungate tubes for 24 hr at 37°C and 300 rpm. Each day thereafter for 5 days, cells were diluted 1:1000 into 15 ml anaerobic LB in a sealed hungate tube containing the respective antibiotic and grown for 24 hr at 37°C and 300 rpm.

To determine the MIC on each day, an aliquot of cells from each treatment condition was diluted 1:10,000 into LB and dispensed into 96-well plates (100 μl total volume per well) containing various concentrations (ten replicates per drug concentration) of norfloxacin, ampicillin, kanamycin, tetracycline, or chloramphenicol. Plates were incubated at 37°C and 300 rpm for 24 hr, after which time the optical density at 600 nm (OD_{600}) was measured using a SPECTRAFluor Plus (Tecan; Männedorf, Switzerland). The median OD_{600} was calculated for each drug concentration, and the MIC was determined as the concentration that inhibited 90% of growth based on OD_{600}. Fold change in MIC was determined by dividing the treated MIC on each day by its respective MIC from day 0.

Determination of MIC Variability and Multidrug Resistance

Wild-type *E. coli* were grown for 5 days in the presence of 1 μg/ml ampicillin or no drug (untreated) as described above. These long-term-treated cultures were diluted 1:1000 into 25 ml LB in 250 ml flasks and grown for 3 hr at 37°C and 300 rpm. Aliquots (1 ml) were plated onto LB-agar plates containing 300 ng/ml norfloxacin, 7.5 μg/ml ampicillin, 15 μg/ml kanamycin, 8 μg/ml tetracycline, and 25 μg/ml chloramphenicol, respectively, and grown for 24 hr at 37°C. Approximately 100 ampicillin-treated colonies from each primary drug selection were purified by streaking them onto LB-agar plates containing the same selective antibiotic. Double the volume of untreated control cells were plated for primary resistance selection as compared to the ampicillin-treated cells, and these untreated colonies were also purified as described above. Plates were placed at 37°C for 24 hr; these strains were then transferred via replica plating onto LB-agar plates containing norfloxacin, ampicillin, kanamycin, tetracycline, or chloramphenicol. Cross-resistance for each primary antibiotic selection following the 5 day ampicillin treatment or the no-drug treatment was determined after 24 hr of growth at 37°C by counting the colonies that displayed growth on the various drug-containing replicated plates.

The MIC of 44 of the above isolates and the MG1655 control strain were determined for ampicillin and norfloxacin, respectively. Overnight cultures of each strain were diluted 1:10,000 into 100 μl LB plus varying concentration of antibiotic (four replicates per strain per drug concentration) in 96-well plates. Plates were incubated at 37°C and 300 rpm for 24 hr, after which time the OD_{600} was measured using a SPECTRAFluor Plus (Tecan). The median OD_{600} was calculated for each drug concentration, and the MIC was determined as the concentration that inhibited 90% of growth based on OD_{600}.

Wild-type *S. aureus* were grown for 5 days in the presence of 35 ng/ml ampicillin or no drug (untreated) as described above. *E. coli* clinical isolate NCDC C771 was grown for 5 days in the presence of 1 mg/ml ampicillin or no drug (untreated) as described above. These long-term-treated cultures were diluted 1:1000 into 25 ml LB in 250 ml flasks and grown for 3 hr at 37°C and 300 rpm. For *S. aureus*, 1 ml aliquots were plated onto LB-agar plates containing 3 μg/ml norfloxacin, 7.5 μg/ml ampicillin, 15 μg/ml kanamycin, 8 μg/ml tetracycline, and 25 μg/ml chloramphenicol, respectively, and grown for 24 hr at 37°C. For NCDC C771, 1 ml aliquots were plated onto LB-agar plates containing 400 ng/ml norfloxacin, 8.5 μg/ml ampicillin, 20 μg/ml kanamycin, and 15 μg/ml chloramphenicol. Tetracycline cross-resistance was not quantified for NCDC C771, as this strain is resistant to tetracycline (MIC > 35 μg/ml). Approximately 100 ampicillin-treated colonies from each primary drug selection were purified by streaking them onto LB-agar plates containing the same selective antibiotic. An equal volume of untreated *S. aureus* or the *E. coli* clinical isolate cells were plated for primary resistance selection as compared to the ampicillin-treated cells, and these untreated colonies were also purified as described above. The remainder of the cell growth and cross-resistance determination was performed as described above for wild-type *E. coli*.

Sequencing of Ampicillin-Treated, Norfloxacin-Resistant, or Kanamycin-Resistant Mutants

Six ampicillin-treated, norfloxacin-resistant isolates and six ampicillin-treated, kanamycin-resistant isolates from the cross-resistance experiment described

above, as well as the untreated MG1655 control strain, were grown to a cell density of approximately 10^9 cfu/ml. Genomic DNA was extracted from each sample using a QIAGEN genomic DNA extraction kit according to the manufacturer's instructions. Primers from IDT (Coralville, IA) (Table S1) were utilized to PCR amplify, using Phusion DNA Polymerase (Finnzyme; Espoo, Finland), the regions surrounding *gyrA*, *gyrB*, *tolC*, *acrA*, *marRA*, *ampC*, *rpsL*, *icdA*, *iscR*, *sdhB*, *arcA*, and *cpxR*. These samples were sequenced by Agencourt Bioscience Corporation (Beverly, MA) using primers from IDT (Table S1). Sequences were analyzed using Clone Manager 7 (Scientific & Educational Software; Cary, NC) and Sequence Scanner v1.0 (Applied Biosystems; Foster City, CA).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Results, Supplemental References, two figures, and one table and can be found with this article online at doi:10.1016/j.molcel.2010.01.003.

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