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Antibiotic-Induced DNA Cleavage: A Role of Restriction-Modification Systems

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The endonuclease and methyl-transferase enzymes of type II restriction modification (RM) systems function independently. Type II RM systems can induce programmed cell death by the same mechanism as addiction modules such as mazEF. The mazEF system has previously been shown to induce cell death when protein synthesis is inhibited. In the current work we tested whether cell death due to protein synthesis inhibition could be mediated by a type II RM system in *Escherichia coli*. We monitored cell death through the detection of DNA cleavage using pulsed field gel electrophoresis at several time points after the addition of antibiotics. We found DNA cleavage and degradation after antibiotic treatment in RM positive strains, but not in the RM negative strain tested. These results suggest that chromosome cleavage may be a mechanism of cell killing by antibiotics.

Type II restriction modification (RM) gene complexes are prevalent on bacterial chromosomes (7), and are typically very stable genetic elements (9). Type II RM systems differ from types I and III in that the restriction endonuclease and methyl-transferase enzymes may function independently (20). This feature allows type II RM systems to facilitate programmed cell death by an analogous mechanism as post-segregational killing systems or 'addiction modules' (16).

The RM system is made up of two components: a methyl-transferase and a restriction endonuclease. The methylase 'protects' the cell from death by ensuring that newly synthesized DNA is methylated, whereas the endonuclease 'toxin' functions to cut DNA if it is not methylated at its recognition site. Cleavage of the cellular DNA will result in cell death (9) and the endonuclease only needs to recognize and cleave one unmethylated site in order for this to occur (7). If a cell loses the genes for this system, daughter cells will still inherit the component proteins. Eventually through further cell divisions or protein turnover the methylase may not be able to modify all of the sites required to protect the chromosome (7, 9, 16). In experiments with temperature sensitive promoters for the RM genes, chromosome degradation was observed two hours after expression of the type II RM system was inhibited (16). In other addiction modules such as the *mazEF*, the MazE anti-toxin is known to have a half-life of 30 minutes (4) and is completely depleted by two-and-a-half hours after its synthesis is inhibited; whereas the MazF toxin is still present at high levels in the cell after 4 hours (1). Chromosome degradation resulting from inhibited expression of the type II RM proteins occurs after 2 hours (16). The half-life of methylase proteins with respect to restriction endonucleases is not yet well characterized, but the time for the restriction activity to occur correlates with the depletion time of the MazE antitoxin.

A type II restriction endonuclease cuts DNA on both strands causing double strand breaks (7, 16). These enzymes usually recognize a specific symmetric sequence of 4 to 8 nucleotides in length and cut either within this sequence or a specific distance from it (20). Endonucleases recognizing shorter sequences have more potential cut sites since shorter sequences will generally occur more frequently in the DNA (20).In order to repair double strand DNA breaks in *Escherichia coli*, homologous recombination using both RecA and RecBCD enzymes is required (2). The chromosome repair action, initiated by the RecBCD complex, degrades linear double stranded DNA produced by the endonuclease (6, 7, 11, 21). Null mutations in the recA gene completely abolish the ability of the cell to repair these double stranded DNA breaks via homologous recombination (2).

Antibiotics that inhibit protein synthesis have been used for many years to kill bacteria; however, the underlying mechanism of killing and the interaction between the antibiotic and the target are not well characterized (10, 17). Antibiotics that inhibit transcription or translation, including chloramphenicol, are able to cause cell death mediated by the mazEF system (17, 19). The present study was conducted to determine if the mechanism of antibiotic-induced cell death can be mediated through the action of type II restriction endonucleases. Wild-type *E. coli* and RM deficient strains were used to address this possibility. Strains with null mutations in the *recA* gene were also used to assess the involvement of the double-strand DNA break repair system. Both intact and cleaved huge chromosomes were detected using pulsed-field gel electrophoresis. Degradation was also detected at later times after antibiotic

treatment for the restriction-modification positive strains. Our results indicate that chromosome cleavage may be a mechanism of cell killing by antibiotics.

MATERIALS AND METHODS

Preparation of Growth Media and Stock Solutions. Modified Luria broth (0.4% glycerol) was prepared as previously described (18). Glycerol was included as an extra carbon source to optimize bacterial growth and obtain higher cell concentrations. Stock solutions of kanamycin and chloramphenicol were prepared as previously described (18). Reagents used for pulsed field gel electrophoresis (including the preparation of agarose plugs) were prepared as per manufacturers instructions.

Bacterial Strains. Three different *Escherichia coli* strains were used: CG76 (Su^+ (E or F), *lac*$^-$ *(?), thi*$^-$ *(?), recA, mutD*); B23 (K-12); and HB101 (F^- *hsdS20*(r^-_B m^-_B), r*ecA13, ara-14, proA2, lacY1, galK2, rpsL20*(Sm^r), xyl-5, mtl-1, supE44, l^-, *leu*.-). All strains were provided by Dr. William Ramey, Department of Microbiology and Immunology, University of British Columbia.

Preparation for Treatment of Cells with Antibiotics. Overnight cultures of each strain, grown in modified Luria broth (18) were inoculated into modified Luria broth (18) for an initial concentration of 5 x 10^7 cells/mL. Cultures were then incubated in a 37 °C water bath with mild aeration.

Treatment of Cells with Antibiotics. Each culture was separately treated with chloramphenicol and kanamycin. The antibiotic was added once each culture reached a turbidity of 0.4 OD_{660}. Chloramphenicol was added to a final concentration of 40 µg/mL. Kanamycin was added to a final concentration of 100 µg/mL.

Generation of Growth Curve. Following the inoculation of Luria broth with overnight culture, turbidity readings (OD_{660}) were taken at various time points. All turbidity readings were taken with an Ultrospec 3000, UV/Visible spectrophotometer (Biochrom).

Pulsed Field Gel Electrophoresis. Two percent agarose (CleanCut™, Bio-Rad) plugs were prepared as per manufacturers instructions. Final plug concentration was 5 x 10^8 cells/ml. Plugs were loaded into a 1% agarose gel prepared in 0.5x TBE buffer, which was also used as running buffer. Plugs containing the chromosomes of the yeast *Saccharomyces cerevisiae* were used routinely as molecular weight standards (Bio-Rad). The gel was run for 24 hours at 14 °C, with a voltage of 6 V/cm and a switch time of 60-120 seconds.

Staining and Photography of Gels. Gels were stained with ethidium bromide for at least 48 hours and then photographed.

RESULTS

Chromosome Cleavage and Degradation. In order to study the effect of the RM system on cleavage of *E. coli* chromosomal DNA, linear DNA was detected *in vivo* using PFGE. Cell lysis was performed in agarose plugs and the gels were stained with ethidium bromide to visualize the DNA (Fig. 1). Under the conditions used the majority of uncleaved chromosomal DNA from the cells remained in the wells.

Smears in the lower molecular weight regions of the gels were taken as evidence of DNA degradation and/or fragmentation (7, 13). Large circular DNA, being intact bacterial chromosomes, remained in the wells whereas huge (>700kbp) linear DNA appeared as bands just below the wells. These bands represent chromosomes that were: cleaved at few sites, that had undergone only weak degradation, and that were partially restored from cut fragments (7, 13).

In the rm^+/rec^+ strain (B23 wt, Fig. 1A) we detected faint bands representing huge linear DNA after treatment with both antibiotics; but more were present for the chloramphenciol-treated cells. Smears of degraded DNA were observed in the lower molecular weight region of the gel and were more pronounced in the chloramphenicol treated cells. They appear to increase over time after treatment with both antibiotics. No cleavage was observed in the untreated cells (lanes 1 and 7); however, some degradation was detected as smearing in the cells prior to treatment with kanamycin (lane 7).

Faint bands corresponding to large linear DNA were detected for the $rm^+/recA^-$ strain (CG76, Fig. 1B) treated with chloramphenicol for all time-points except two hours post-antibiotic addition. No cleavage was observed in the untreated cells (lanes 1 and 8). Cleavage was detected as a large linear fragment for the kanamycin-treated cells (lanes 9 through 13). Degraded DNA was visualized as smearing at the later time points for both antibiotics (lanes 4-6 and 12-13). Intact chromosomes remained in the wells (all lanes).

When we compared the rm^+/rec^+ strain (B23, Fig. 1A) with the $rm^+/recA^-$ strain (CG76, Fig. 1B), we found that the rm^+/rec^+ strain had more intense huge linear DNA bands than the $rm^+/recA^-$ strain. The DNA smears detected in the $rm^+/recA^-$ strain appear to be less intense than in the rm^+/rec^+. These also increased over time in the rm^+/rec^+ strain but remained constant in the $rm^+/recA^-$ cells.

In the $rm^-/recA^-$ strain (HB101, Fig. 1C) we did not observe any huge linear DNA or any DNA degradation. Huge intact chromosomes remained in the wells (lanes 1-7 and 9-13).

DISCUSSION

Our results demonstrate that RM systems may mediate cell killing as a consequence of antibiotic treatment. No cleavage or degradation of DNA was observed for the RM deficient strain of *E. coli* (HB101) after antibiotic

treatment. In contrast, both of the strains with functional RM systems showed some DNA degradation and / or cleavage after treatment with antibiotics.

In the *rm*$^+$*/rec*$^+$ strain our results agree with our expectations. Since the RM system is functional, the chromosome is cleaved due to a decrease of the methylase after treatment with the antibiotic. This results in the detection of the large linear fragments represented by the bands directly below the wells. The Rec repair system is also functional in this strain, therefore little degradation is observed initially (compared to the *rm*$^+$*/recA*$^-$ strain). More DNA degradation was observed in later time points, and these smears increased over time after prolonged antibiotic exposure. This is due to the fact that the Rec repair system, along with ligase repair is unable to keep up with the increase in restriction cuts in the chromosome, thus resulting in an increase in chromosomal degradation (8).

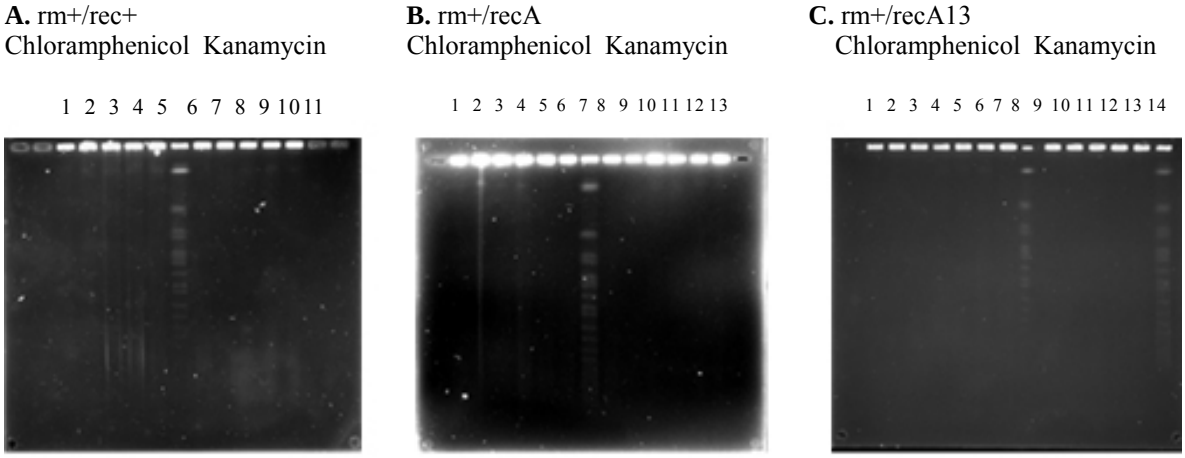

Figure 1. Pulsed field gel electrophoresis from *E. coli* strains cultured in liquid LB media at 37^{o}C. (**A**) Strain B23 treated with 40 µg/ml Chloramphenicol (lanes 1-5) or 100 µg/ml Kanamycin (lanes 7-11), and analyzed at 0, 22, 49, 84, and 121 minutes. (**B**) Strain CG76 treated with 40 µg/ml Chloramphenicol (lanes 1-6) or 100 µg/ml Kanamycin (lanes 8-13), and analyzed at 0, 19, 44, 90, and 120 minutes. (**C**) Strain HB101 treated with 40 µg/ml Chloramphenicol (lanes 1-6) or 100 µg/ml Kanamycin (lanes 7, 9-13), and analyzed at 0, 10, 20, 47, 96, and 128 minutes.

According to Handa *et al.* (7), *recA* mutants show more extensive cell death and accumulate more large cleaved chromosomes. We expected to see more of the large linear DNA and more smearing in the *rm*$^+$*/recA*$^-$ strain than in the *rm*$^+$*/rec*$^+$ or *rm*$^-$*/recA*- strains. In the *rm*$^+$*/recA*$^-$ strain we also expected to see an increase in smearing compared to the other two strains because of the inability to repair broken chromosomes, leading to their extensive degradation (7). Since RecA could not perform homologous recombination, the cells would be unable to repair the cleaved DNA (2, 12, 14). Over time this degradation would increase due to the continued activity of the restriction endonuclease and the exonuclease activity of RecBCD (15). However, our results did not support this. We saw only faint bands corresponding to large linear DNA, less than that observed in the *rm*$^+$*/rec*$^+$ strain. Also, smears of degraded DNA were about the same intensity as the *rm*$^+$*/rec*$^+$ strain. Our results probably did not strongly support our hypothesis due to an inability to optimize the parameters of the PFGE. In order to achieve better results it is important to repeat the experiment testing different parameters of the PFGE such as the concentration of DNA in the agarose plug (to allow better migration of linear DNA from the wells into the gel), cell concentration, lysozyme treatment (in order to get better DNA isolation within the plugs), voltage, running time, gel thickness, and other variables (5). Additionally, since we are most likely isolating large chromosomal fragments as the result of one or few cuts it may be desirable to increase the pulse-switch time for the duration of running the gel to more specifically resolve larger fragments (5). It is of note that the problems of band visibility and the observation of intact chromosomes remaining in the wells were also encountered by *Handa et al*. (7) under PFGE conditions near-identical to those used in the present study.

Since the HB101 strain is *rm*$^-$*/recA*$^-$, we did not expect to observe any DNA cleavage or degradation. Our results agreed with these expectations, as the gel showed the presence of only intact chromosomes. Cleavage of the

chromosome was not seen in this strain, suggesting that there is no other system present within the cell that can cause DNA cleavage. This supports that the DNA cleavage seen with the other two strains is indeed due to the RM system rather than another unknown system.

For both *rm*$^+$*/rec*$^+$ and *rm*$^+$*/recA*$^-$ strains, more intact chromosomes were seen in the wells of the chloramphenicol-treated bacteria. Because chloramphenicol is a bacteriostatic antibiotic, DNA replication is inhibited so that no new rounds of replication occur after treatment (3). Since inhibition of protein synthesis by chloramphenicol is not lethal at the dosage used, enough methylase may be produced which would prevent DNA cleavage by the restriction endonuclease. In contrast, kanamycin is bacteriocidal, therefore, protein synthesis inhibition by this antibiotic must directly or indirectly lead to cell death. Less methylase would be expected to be synthesized in cells treated with this antibiotic than in chloramphenicol-treated cells if cell death is in fact mediated by the RM system. Thus, more extensive DNA cleavage or degradation was expected with the kanamycin-treated cells. This was observed for the kanamycin treated *rm*$^+$*/rec*$^+$ cells. However, the opposite was observed for the *rm*$^+$*/recA*$^-$ strain. In a duplicate experiment using the *rm*$^+$*/recA*$^-$ strain, more DNA degradation was seen with the kanamycin-treated cells compared with those treated with chloramphenicol (data not shown). In addition, no large linear DNA was detected for cells treated with either antibiotic. Verification of these different results will be important for future studies.

It is difficult to determine the extent of DNA cleavage and degradation in the *rm*$^+$*/recA*$^-$ due to difficulties in staining the gel. Immediately after exposing the gel, linear DNA fragments were visible directly below the wells for both the chloramphenicol and kanamycin treated cells. However, the bands began to fade and the banding pattern was not captured on film. Thus, figure 1B, does not accurately reflect what was on the gel. Before the bands faded, it was observed that the chloramphenicol treated cells had more smearing than the kanamycin treated cells, as seen in the *rm*$^+$*/rec*$^+$ strain.

Since *E. coli* also harbors the *mazEF* addiction module, and this system can mediate cell death after treatment with protein synthesis inhibitors (17, 19), it is uncertain how large a role RM systems may play in mediating cell death. Furthermore, the cellular target of the MazF toxin is not yet known (4). The targets of restriction endonucleases are extremely well characterized. The DNA cleavage we observed after antibiotic treatment in the *rm+* cells, and not in the RM-deficient strain favor a mechanism of cell death induced by this system.

In conclusion, cleavage and degradation of *E. coli* chromosomal DNA which occurs after the addition of antibiotic may be mediated by the RM system. Moreover, our results suggest a novel mechanism for antibiotic-induced cell death.

FUTURE EXPERIMENT

Since the greatest limitation in the current study was the low visibility of bands on the gels, further studies should be conducted to optimize the assay conditions to give better results and verify the limited observations noted in the present work. This may involve using an exogenous rare cutting restriction enzyme to cleave the chromosome and comparing the restriction patterns of the RM-containing and RM-deficient strains with or without antibiotic treatment. This would create more chromosome fragments and allow their migration into the gel which would help to increase visibility of bands. Alternately, a different assay to test the involvement of RM systems in cell death could be developed. Currently the *in vivo* half-lives of the proteins comprising the RM system are not known. Therefore a study to monitor the methylase and endonuclease concentration over time following the addition of antibiotic would be useful. A significant decrease in the methylase, and not in the endonuclease, would strengthen our findings. Additionally, different protein synthesis inhibitors and varying concentrations of them could be tested to determine the most efficient combination for inducing DNA cleavage. In the present study we used different strains of *E. coli* to test our hypothesis. A better control would be to use the same strain with modifications. A knock-out could be created from the wild-type, or an expression vector could be added to an RM-deficient strain to better demonstrate the direct involvement of the RM system.

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