

Studies on Disinfectant Resistance in *Escherichia coli*

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Abstract

Do bio-warfare (BW) and clinical pathogens evolve and acquire resistance to disinfectants, i.e. Lysol®? A clinical surrogate, *Escherichia coli* was used in these studies. *E. coli* cells were grown in the presence or absence of Lysol®, an all-purpose cleaner, EPA reg# 777-89 (Ready To Use [RTU] 1:16 dilution). The parent strain was sensitive to presence of 1.6% of RTU strength Lysol®. Through progressive sub-culturing, a resistant strain, LR50 was derived, which was resistant to 50% of the RTU strength Lysol®. A 30-fold increase in resistance to Lysol® illustrates bacterial cells' genome plasticity and adaptive phenomenon. In a separate series of experiments, *E. coli* cells adaptive resistance to Germ-X®, a hand sanitizer, was also observed. Relative to parent strain, GR17 – a resistant strain was derived capable of growing in the presence of 17%.

The phenotype of LR50 was confirmed by sub-culturing in Tryptic Soy Broth (TSB) five times and then confirming its resistance phenotype in the presence of 50% Lysol®. Antibiotic resistance of LR50 and the parent strain was tested by measuring zones of inhibition. LR50 was resistant to 5 µg rifampin. Biochemical characterization revealed the presence/absence of specific polypeptides unique to the LR50. Genomic sequencing was done and there are some single nucleotide polymorphisms (SNPs) that are unique to LR50.

Background

The recent emergence of a new resistance mechanism in the "superbug" phenomenon and its spread across bacterial species illustrates the microbial adaptability to sub-lethal exposure to antibiotics. The increasing use of disinfectants in clinical and household settings has raised serious concerns for acquisition of resistance in bacterial cells. Mechanisms underlying these two phenomena may be quite different. A clearer understanding of the biochemical and genetic adaptation is expected to offer new insight into counter-measure development.

Penicillin was first used to treat bacterial infections in the 1940's. By the 1950's, penicillin-resistant strains of *Staphylococcus aureus* were common. Methicillin was first introduced in 1961 to treat infections with such cells. Within one year, Methicillin-resistant (MRSA) strains were encountered. Today, strains of MRSA are resistant to a host of other antibiotics, including Vancomycin. Bacterial cells evolve resistance to antimicrobial use, the short generation times and large population size of bacteria help boost this evolution.

Disinfectants are commonly used in water treatment plants and for cleaning surfaces in hospital settings and medical treatment facilities. Household use of disinfectants is exploding, an over two billion dollar industry and still growing. Disinfectants typically kill 99.999 percent of pathogens within 5-10 minutes. Chlorine and quaternary ammonium compounds (quats) are common key ingredients. The general mechanisms of disinfectants include cell membrane destruction, interference with key biochemical function, blockage of nutrient uptake and prevention of waste products. Ever-increasing disinfectant use challenges bacterial populations to evolve into resistant isolates.

Mechanisms for disinfectant resistance of bacterial cells include gelatinous exopolysaccharides secretions to form biofilms by some species. Recruitment of an efflux pump to selectively export the disinfectant is another possible mechanism. Some bacteria adaptation may involve alteration in gene expression of novel transporter proteins.

Materials and Methods

E. coli, which is a gram negative, facultative anaerobic, rod-shaped bacteria, was used in present study.

The disinfectants used were Lysol® brand disinfectant all-purpose cleaner, 4 in 1 and Germ-X®, a hand sanitizer. The ingredients in Lysol® are alcohols, C12-16, ethoxylated (2.5-10%), Alkyl (50%C14, 40%C12, 10%C16) dimethyl benzyl ammonium chlorides (1-2.5%), and ethanol (0.1-1%). This disinfectant has both alcohols and quaternary ammonium chlorides. Alkyl dimethyl benzyl ammonium chloride attaches to the bacteria and causes the cytoplasmic membrane to leak, which damages and then kills the bacterial cell.

The active ingredients in Germ-X® are 59% ethyl alcohol and 3% isopropyl alcohol.

The RTU concentration recommended by the manufacturer is 1:16 for Lysol®, which was further diluted 1:10 to a working stock, and was regarded as 100%. The RTU concentration of Germ-X® is 100% strength.

Tryptic Soy broth was used as the diluent in all the experiments.

The RTU Lysol® was further diluted by 1:10 to a working stock.

The parent strain of *E. coli* was screened by the Micro-Titer plate assay for sensitivity. After this, the sensitivity percent was determined.

E. coli cells were grown over 6 months in increasing Lysol® concentrations. Growth curves were then performed for the parent strain (PS) *E. coli*, the Lysol® resistant (LR) strain in both TSB and 50% of Lysol®. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels were run to compare the proteins in both the parent strain and the LR strain. The two strains were also examined by microscopy to compare the cell types.

For genomic sequencing, the samples were prepped using the Nextra sequencing kit and sequenced on an Illumina HiSeq 2000. The run configuration used was 2x100; meaning reads were produced in pairs, with each pair being 100 bases long. Greater than 5 gigabases were produced for each sample. Data was analyzed using Bowtie, SAMtools, and CLC Bio Genomics Workbench.

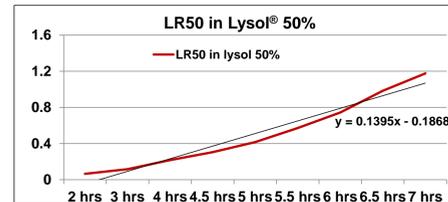


E. coli cells grown in 50% Lysol® RTU are referred to as LR50. The resistance phenotype in the derivative cells is genetically stable as evident by retention of this phenotype even after five sub-cultures in the absence of Lysol®. The doubling times for parent strain and LR50 isolates is very similar, i.e. 60 minutes.

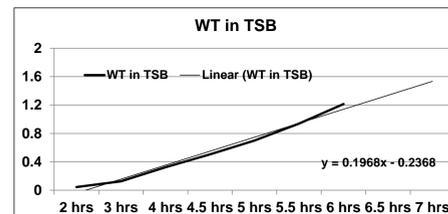
Data



Doubling time of LR50 in TSB is 60 minutes



Doubling time of LR50 in 50% Lysol® is 65 minutes

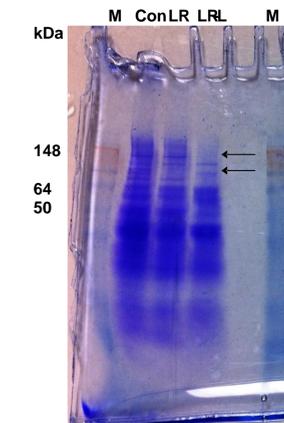


Doubling time of parent strain is 60 minutes

Single Nucleotide Polymorphisms (SNPs) Unique to LR50

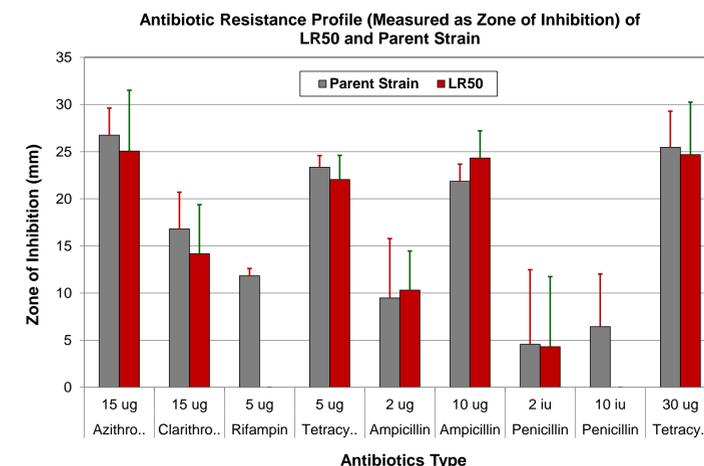
SNP	Protein
<i>acrB</i>	multidrug efflux system pump
<i>mdtB</i>	multidrug efflux system, subunit B
<i>yfjW</i>	inner membrane protein
<i>yghB</i>	inner membrane protein

Protein Gel



M = protein markers
Con = Control
LR = Lysol®-Resistant Strain in TSB
LR-L = Lysol® Resistant Strain in 50% Lysol®

- The LR strain grown in 50% Lysol® is missing a polypeptide ~130 kDa, which is present in the LR strain and control strain grown in TSB
- A polypeptide of ~100 kDa is uniquely present in LR50, when grown in the presence of Lysol®
- Alterations in gene expression appear to be the basis for the LR50 phenotype



Discussion and Conclusions

The growth and sub-culture of *E. coli* cells (LR – Lysol®-resistant) were continued for over 6 months in increasing concentrations of Lysol®, until the cells were able to grow in the presence of 50% of the RTU Lysol®. The phenotype of LR *E. coli* cells, which is referred to as LR50, was confirmed by sub-culturing in TSB (absence of Lysol®) for 5 times and then growing in the presence of Lysol®. The LR50 phenotype was confirmed by culturing glycerol frozen stock in the presence of 50% Lysol® in independent runs.

Growth curves were performed with parent strain *E. coli* cells grown in TSB, LR50 cells grown in Lysol® 50%, and parent strain *E. coli* grown in Lysol® 50%. The doubling times for both parent strain and LR50 in TSB were about 60 minutes. The doubling time for LR50 in 50% Lysol® was 65 minutes. As expected, parent strain *E. coli* did not grow in 50% Lysol®.

Preliminary results suggest that there are differences in protein levels of the parent strain and LR50, suggesting alterations in gene expression as a basis for LR50 growth in 50% Lysol®. The LR strain grown in 50% Lysol® is missing a polypeptide of ~130 kDa, which is present in the LR strain and the parent strain grown in TSB. The LR strain grown in 50% Lysol® has a polypeptide of ~100 kDa that seems to be missing in both the LR strain grown in TSB and the parent strain.

Nineteen antibiotics were tested against both LR50 and the parent strain. Compared to the parent strain, LR50 had no zone of inhibition around rifampin 5 µg and penicillin 10 iu. Only LR50 was observed to be resistant to two antibiotics, rifampin 5 µg and 10 iu penicillin.

DNA was isolated from both the parent strain and LR50. The genome sequence of LR50 and the parent strain was compared. The following SNPs were unique to LR50, *acrB*, multidrug efflux system protein, *mdtB*, multidrug efflux system, subunit B, *yfjW*, inner membrane protein, and *yghB*, inner membrane protein.

The multidrug efflux system protein altered in LR50 and not in the parent strain could be the likely cause for LR50 to be resistant. Efflux pumps are transport proteins that get rid of toxins from within the bacterial cells. Future work is needed to show if LR50 recruited an efflux pump as a plausible mechanism for observed resistance. Studies exploring adaptive potential of other pathogenic *Acinetobacter baumannii*, *Pseudomonas diminuta*/ *Staphylococcus aureus* against disinfectants, such as Germ-X® and quats, are highly desirable.

Acknowledgements: The authors thank the Army's Environmental Quality Basic Research program managed by ARDEC, in Picatinny, NJ for funding of this project. We also acknowledge Savannah Maggio for laboratory preparatory work and Dr. Nicole Rosenzweig for facilitating the genome sequencing. The views expressed in this presentation are those of the authors and do not necessarily reflect the official policy or position of the Department of Defense or the US Government.



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