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CONTINUAL PASSAGE OF *STAPHYLOCOCCUS EPIDERMIDIS* IN SUBINHIBITORY LEVELS OF THE BIOCIDE TRICLOSAN RESULTS IN A MARKED INCREASE IN THE MINIMUM INHIBITORY CONCENTRATION, ANTIBIOTIC RESISTANCE, AND ETHIDIUM BROMIDE RESISTANCE

being

A Thesis Presented to the Graduate Faculty

of the Fort Hays State University

in Partial Fulfillment of the Requirements for

the Degree of Master of Science

by

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This Thesis for

The Master of Science Degree

By

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Has been approved

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ABSTRACT

Triclosan is a multi-purpose biocide that is used in many personal care products, including antibacterial handsoaps and toothpastes. The wide usage of triclosan fosters its dispersal into the environment, which might contribute to the ability of microorganisms to become resistant to triclosan in addition to certain other biocides and clinical antibiotics.

The aim of this study was to evaluate whether long-term exposure of two strains of *Staphylococcus epidermidis* to subinhibitory concentrations of triclosan would select for resistant mutants, and whether their ability to form polysaccharide biofilms lends to this resistance. This study also aimed to determine whether a mutation in the triclosan target was responsible for resistance, and to determine whether these mutants could exhibit cross-resistance to chlorhexidine and clinical antibiotics. In addition, efflux capability was assessed as a presumable resistance mechanism.

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I thank the Kansas IDeA Network of Biomedical Research Excellence for funding my research. I thank Dr. Greg Somerville at the University of Nebraska for donating the two *Staphylococcus epidermidis* strains used in this study.

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PREFACE

The figures and literature cited in this thesis were written according to the format of the Journal of Bacteriology, published by the American Society for Microbiology, to which it will be submitted for publication.

INTRODUCTION

Staphylococci

The Scottish physician Sir Alexander Ogston identified the bacterial genus *Staphylococcus* in 1880, as it was one of the primary causative agents associated with wound infections (37). Sir Alexander named *Staphylococcus* after he observed its characteristic grape-like clusters under a microscope (37). Staphylococci are Gram-positive cocci that are non-flagellate, non-motile, non-spore forming, facultative anaerobes that produce the enzyme catalase (1).

Staphylococcus is commonly divided into two distinct groups: those that produce the enzyme coagulase, and those that do not (1). Jacques Loeb first reported coagulase activity in 1904 (22). Loeb's method of observation is now referred to as the tube coagulase test, which led to the further examination and characterization of *Staphylococcus aureus* in 1934 (22). Coagulase is an enzyme that binds to prothrombin, and initiates the polymerization of fibrin, which results in the coagulation of blood plasma (1). *Staphylococcus aureus* is a coagulase-positive organism relevant to the field of medicine (1). The medical relevance of *Staphylococcus aureus* is largely due to its multiple virulence factors including toxic shock syndrome toxin-1, alpha-toxin, emetic pyrogenic superantigens, and enterotoxins (1) *Staphylococcus aureus* was isolated in 1884 by German scientist Anton Rosenbach (36). Rosenbach also distinguished between *Staphylococcus aureus* and *Staphylococcus epidermidis* by describing two pigmented colony types (36). The pigments led to his appropriately proposed nomenclature: *Staphylococcus aureus* so named for its golden color, and *Staphylococcus albus* for its white color. *Staphylococcus albus* is now known as *Staphylococcus epidermidis* (36).

Coagulase-Negative Staphylococci

Members of the genus *Staphylococcus* that do not produce coagulase are referred to as coagulase-negative staphylococci (CoNS). CoNS are often used in the food processing industry as starter cultures for fermented food products such as fermented sausages (22). Such organisms include *Staphylococcus xylosus, Staphylococcus carnosus, Staphylococcus succinus,* and *Staphylococcus equorum* (22).

Other CoNS are found naturally living in the mucous membranes and on the surfaces of warm-blooded birds and animals, including humans (21). Coagulase-negative staphylococci are often considered to be beneficial as they are used in the food processing industry, and because they exist as normal floral symbionts. However, CoNS are opportunistic pathogens, especially in immunocompromised, long-term hospitalized, and critically ill patients (22).

Common CoNS that have the ability to produce infection in humans include *Staphylococcus saprophyticus* and *Staphylococcus epidermidis. Staphylococcus saprophyticus* is a common cause of urinary tract infections in sexually active females (1). *Staphylococcus epidermidis*, which is most often associated with medical prosthetic devices, is the most common CoNS of concern (1). Infection can occur upon implantation of a device by either the seeding of the device during a prior bacteremia or by gaining access to the lumina of catheters and shunts (1).

Pathogenesis of *Staphylococcus epidermidis*

Staphylococcus epidermidis, which is the most frequently isolated species of CoNS, is the leading cause of infections related to prosthetic medical devices (49). The

ability of *Staphylococcus epidermidis* to cause infection is due to virulence factors such as delta-toxin (47). *Staphylococcus epidermidis* is also frequently able to resist the action of antibiotics due to its ability to form viscous extracellular polysaccharide biofilms on surfaces (27). Multiple factors facilitate the initial adherence of *Staphylococcus epidermidis* to prosthetic devices, including macromolecular components in body fluids such as blood, urine, saliva, and mucus (6). Other nonspecific physiochemical variables for adherence include Van der Waals forces, surface tension, temperature, and electrostatic interactions (12). *Staphylococcus epidermidis* also has surface proteins including SSP-1 and SSP-2, which function in the adherence of the cells onto polystyrene surfaces (46). The surface protein function is largely due to their organization into fimbria-like structures (46). Once adherence has occurred, the proliferation stage commences, where the production of extracellular polysaccharides and polysaccharide intercellular adhesin (PIA) is upregulated cementing the cells to each other and to the surface (6). PIA is a linear ß-1,6-linked glucosaminoglycan which is synthesized by enzymes encoded by the *ica* operon (33). PIA provides extra adhesion and encases the entire bacterial population, acting as a shield against the host defense systems and externally administered antimicrobial agents (33).

A mature biofilm is comprised of several layers and reveals groups of microcolonies, which are separated by fluid-filled channels (33). These channels are thought to facilitate distribution of nutrients and oxygen throughout the biofilm in addition to the removal of metabolic waste (17).

Detachment of cells from a biofilm is the combined effect of cell viability, growth patterns, and shear stress (51). *Staphylococcus epidermidis* secretes delta-toxin, which

lyses erythrocytes in mammalian hosts, acts as a detergent during biofilm detachment (47). The accessory gene regulator (*agr*) quorum sensing system is also thought to function in biofilm detachment by downregulating surface protein expression and upregulating exoenzyme and toxin expression (37). The *agr* quorum sensing system has been observed as being expressed only by the outer, most exposed, layers of the biofilm (48).

Clinical problems that have arisen due to the formation of biofilms on indwelling medical devices are largely due to the fact they are frequent inhabitants of the surface of human skin, mucous membranes, ear canals, and anterior nares. In the past 50 years, *Staphylococcus epidermidis* has become a significant opportunistic pathogen due to its ability to resist certain antibiotics, especially in hospital patients who have received vascular grafts, heart valves, coronary stents, and fracture-fixation implants (10). The ability of *Staphylococcus epidermidis* to resist multiple antibiotics is largely due not only to the ability of the organism to form biofilms, but also to the extensive use of antimicrobials and disinfectants, which exerts selective pressure (33). This selective pressure can potentially lead to the evolution of a multi-drug resistance phenotype.

Antibiotic Resistance Mechanisms

The ability of staphylococci to resist antibiotics continues to escalate as one of the major complications in medical microbiology. Misuse of antibiotics including using them to treat colds, flu, or other viral infections, causes the antibiotics to become less effective against the bacterial agents they were originally intended to treat (31). Less than 3% of *Staphylococcus aureus* strains were resistant to penicillin G when it was first introduced

(1). Over 90% of *Staphylococcus aureus* strains are now resistant to penicillin G (1). This phenomenon illuminates the potential for rapid bacterial evolution resulting in antibiotic resistance. *Staphylococcus epidermidis*, being an abundant inhabitant of human skin, is constantly exposed to multiple forms of selection pressure such as over the counter antibacterial products. This form of opportunity combined with its bountiful genetic flexibility makes *Staphylococcus epidermidis* the perfect contender for the development of resistance. As antibiotic resistance continues to emerge as one of the greatest public health concerns on a global scale, one of the aims of the scientific community is to identify factors that are essential for the virulence of pathogens (29).

There are several known mechanisms used by bacteria to resist antibiotics. Some bacteria produce enzymes that alter the antibacterial agent so it can no longer bind to its target molecule (1). Some bacteria have evolved the ability to alter the molecule targeted by a particular antibiotic (1). Certain bacteria, namely Gram-negative organisms, alter porins, which leads to a decreased uptake of the drug (1). Other organisms use molecular efflux pumps to export antimicrobials out of the cell (1). These efflux pumps have been attributed to the ability of cells to eliminate more than one antibiotic (9).

The resistance mechanisms mentioned above could be evolved independently or acquired on mobile genetic elements via conjugation, transduction, or transformation, which often facilitates the incorporation of multiple resistance genes into the genome or plasmids within the host cell (45).

Enoyl-Acyl Carrier Protein Reductase (Fab1) and Triclosan

Antibiotics seek to inhibit pathways required for a bacterium to survive, yielding either a bactericidal or bacteriostatic effect. An important pathway used by *Staphylococcus epidermidis* is the assembly of fatty acids via the expression of the enoyl-acyl carrier protein reductase gene (*fab1*) (18). The assembly of fatty acids brings together two-carbon units in a cyclic sequence of reactions (18). Fab1 is used to catalyze the final step in each cycle (18). Fab1 also plays a regulatory role in determining the rate of fatty acid synthesis. Inhibitors of this step in the fatty acid synthesis pathway such as hexachlorophene and triclosan are thus effective antibacterial agents (Fig. 1) (18).

2-Hydroxyphenylethers make up a group of compounds exhibiting a broad antimicrobial activity spectrum (7). Of these compounds, 2,4,4'-trichloro-2'-hydroxydiphenyl ether, more commonly referred to as triclosan (Fig. 2), is the most potent and widely used (7). Triclosan was first introduced in 1965 and has been shown to be very stable, as it has the ability to resist degradation in both dilute acidic and alkaline solutions (50). Triclosan is a multi-purpose biocide and has been used for more than 30 years in many personal care products, including antibacterial hand soaps, antiseptics, cutting boards, facial cleansers, lotions, and toothpastes (15). This wide and long-term use not only exposes human normal floral organisms to the biocide, but fosters the dispersal of the biocide into the environment, which, as the present study indicated, might explain the ability of microorganisms to become less susceptible to antibiotics and biocides, including triclosan, via either intrinsic or acquired mechanistic adaptations upon exposure (42).

It was once thought the mode of action of triclosan was nonspecific cellular membrane disruption (18). However, it is now known triclosan works by inhibiting enoyl-acyl carrier protein reductase (Fab1) in a broad spectrum of both Gram-positive and Gram-negative organisms which use this enzyme in the elongation cycle of bacterial fatty acid biosynthesis (13). Triclosan, which exhibits the hallmarks of a slow-binding inhibitor, inhibits Fab1 by forming a stable, non-covalent, Fab1-NAD^+-triclosan ternary complex, leading to complete inhibition of bacterial growth and replication (18) (Fig. 1).

Triclosan Resistance

Despite its potent mode of action, there are some bacteria that remain resistant to triclosan. Some of the various mechanisms of conferred triclosan resistance include: decreased influx/membrane permeability, increased target expression, the expression of highly efficient efflux pumps that function to rid the cell of triclosan, target mutation, the production of an enoyl reductase enzyme having a low affinity for triclosan, and the expression of a triclosan degrading enzyme (39, 50). For example, *Pseudomonas aeruginosa* expresses Fab1 but is still resistant to triclosan due to expression of the MexAB-OprM efflux system (7).

Staphylococcus aureus usually is susceptible to triclosan. Triclosan has thus been used in an effort to control the spread of methicillin-resistant *Staphylococcus aureus* (MRSA) in hospitals (24). A study conducted in 2003 suggested the wide usage of triclosan would not select for triclosan resistant MRSA; however, it was found that some MRSA clones might not be as susceptible to triclosan as normal strains (2). Other

laboratory studies have shown mutations in *fabI* and their overexpression correlate to the decreased susceptibility of *Escherichia coli* and *Staphylococcus aureus* to triclosan (13).

A recent study showed repeated *Staphylococcus aureus* exposure to subinhibitory triclosan concentrations resulted in increased resistance to triclosan (24). Triclosan exposure also led to the attenuation of biofilm forming ability, hemolysis, DNase, and coagulase activities (24). These data suggest an increased triclosan resistance could also be associated with reduced pathogenicity (24). Latimer et. al., 2012 used a concentration of 0.0029% triclosan, which is a concentration several orders of magnitude lower than the concentration used in most commercial products. The study presented in this thesis used triclosan concentrations up to 1.5% to simulate the actual effects of using products containing therapeutic concentrations of triclosan as an active ingredient.

Correlation of Triclosan Resistance to Clinical Antibiotic Resistance

In addition to the wide use of triclosan selecting for resistance, one of the major concerns of the overuse of triclosan is its ability to cause resistance to other antimicrobial agents, including traditional, clinical antibiotics. It is thought inappropriate administration of antibiotics can select for more generalized resistance (31). This rationale has been demonstrated in several bacterial strains including *Pseudomonas aeruginosa* and *Escherichia coli* (7). It has also been demonstrated in *Salmonella enterica* and *Mycobacterium smegmatis*, in which resistance to triclosan has also been shown to lead to resistance to the antibiotic isoniazid (4, 7). The prevalence of *Staphylococcus epidermidis*, its constant exposure to triclosan, inherent genetic flexibility, and the

multiple demonstrations of triclosan-mediated cross-resistance to traditional antibiotics in different organisms, suggests *Staphylococcus epidermidis* could demonstrate a profound ability to resist triclosan, which might help mediate cross-resistance to antibiotics with multiple modes of action. To test this rationale, the present study used six antibiotics to represent several of the broad classes of antibiotics, based on mode of action. These were ampicillin and vancomycin, which affect cell wall synthesis, azithromycin, which acts on the 50S ribosomal subunit to interfere with protein synthesis, gentamicin and tetracycline, which also interfere with protein synthesis, but by acting on the 30S ribosomal subunit, and ciprofloxacin, which targets DNA gyrase and topoisomerase IV interfering with nucleic acid synthesis. These antibiotics are chemically classified as β-lactams, glycopeptides, macrolides, aminoglycosides, tetracyclines, and fluoroquinolones respectively (1).

Chlorhexidine

N-(4-chlorophenyl)-1-3-(6-{N-[3-(4 chlorophenyl) carbamimidamidomethanimidoyl] amino} hexyl) carbamimidamidomethanimidamide, more commonly known as chlorhexidine, is an antimicrobial compound often used in such products as surgical scrubs, topical anti-infective agents, and oral rinses (11). Chlorhexidine is effective against a broad range of Gram-positive and Gram-negative organisms and is thought to function by destroying the integrity of the cell membrane and precipitating the cytoplasm (11). This mechanism makes a chlorhexidine resistance phenotype highly unlikely; however, development of stable resistance to chlorhexidine has been observed in strains of *Pseudomonas stutzeri* after being exposed to increasing

concentrations of the agent (44). These resistant strains have also shown reduced sensitivity to antibiotics and biocides such as triclosan (5). Resistance is thought to be associated with cell envelope alterations or the presence of constitutive degradative enzymes (5).

Project Overview

This project sought to determine whether exposure of two different strains of *Staphylococcus epidermidis* to the biocide triclosan could lead to an increased minimum inhibitory concentration. This study also sought to determine whether an increased resistance was made more efficient by the ability of the organism to form a polysaccharide biofilm. This project investigated whether triclosan resistance in *Staphylococcus epidermidis* could be mediated by *fabI* mutation or an increased efflux capability.

With respect to triclosan, this study also aimed to determine whether long-term exposure to subinhibitory triclosan could lead to an increased resistance to the disinfectant chlorhexidine or clinically administered antibiotics.

MATERIALS AND METHODS

Bacterial Cultures

Two *Staphylococcus epidermidis* strains were donated by Dr. Greg Somerville's lab at the University of Nebraska. These strains are SE1457 and SE1457Δica. SE1457 has been genetically altered to overexpress the intercellular adhesion (*ica*) operon, while the *ica* operon has been removed from SE1457Δica to have discernable biofilm positive and negative strains, respectively.

Establishing the Triclosan Minimum Inhibitory Concentration

Unless stated otherwise, all incubations in this work were at 37 °C. Triclosan stock was prepared by dissolving 0.75 g of triclosan in 5.0 mL of 95% ethanol. This is 15% triclosan, which is 100X the normal therapeutic concentration in personal care products, which is 0.15%. This stock solution was diluted by adding 50 µL of the 100X stock to 5.0 mL of tryptic soy broth (TSB). A ten-fold serial dilution scheme was then used to dilute the triclosan to a series from 0.15% to 0.0000015%. Fifty microliters of overnight cultures of both strains of *Staphylococcus epidermidis* were introduced into 5.0 mL of each of the serially diluted triclosan-containing broths. The strains were incubated for 24 hours. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of triclosan in which there was no turbidity.

Establishing the Chlorhexidine Minimum Inhibitory Concentration

A 20% w/v aqueous solution of chlorhexidine gluconate (Alfa Aesar, Ward Hall, MA) was diluted in TSB to 2.0%, which is the typical concentration used in oral rinses and scrubs. A ten-fold serial dilution scheme was used to dilute the chlorhexidine to a series from 2.0% to 0.000002%. The serially diluted tubes were each inoculated with 50 μL of overnight TSB cultures of either SE1457 having been passed in TSB for 70 days, SE1457 having been exposed to subinhibitory triclosan for 70 days, SE1457Δica having been passed in TSB for 70 days, or SE1457Δica having been exposed to subinhibitory triclosan for 70 days. The cultures were incubated for 24 hours, and the MIC of each strain was defined as the lowest concentration of disinfectant at which there was no turbidity.

Cell maintenance

Once the MICs of triclosan were established for each strain, one group of both strains was exposed to a subinhibitory concentration of triclosan for 14 days, while another group of each strain was grown in TSB in the absence of triclosan. In this case, the subinhibitory concentration was 1/10 of the MIC. Each group of cells was incubated for 24 hours, and then 50 μL of culture were passed into 5.0 mL of the appropriate fresh growth medium after each 24-hour incubation period. The triclosan MICs were reevaluated at the end of every 14-day period via the serial dilution method mentioned previously. Whenever an MIC increase was observed, the subinhibitory concentration to which the cells were exposed was increased accordingly such that the cells continued to be exposed to a 1/10 subinhibitory concentration.

Frozen stocks of the unexposed cells and the triclosan exposed cells were prepared each time an increase in MIC was observed. To do this, a sterile 60% glycerol solution was prepared by diluting glycerol with deionized water. The stocks were then prepared by combining 750 µL of overnight culture and 250 µL of the 60% glycerol solution in 1.5 mL microcentrifuge tubes. The resulting stocks were frozen at -80 °C.

Polymerase Chain Reaction

The enoyl-acyl carrier protein reductase gene (*fab1*) was amplified by the polymerase chain reaction (PCR). Primers were designed from the sequence of *Staphylococcus epidermidis fab1* deposited in GenBank. The primers used were Fab1F (5' AGTATCGCATTTGGCGTCGCT 3') and Fab1R (5'GCGTTTTAACGGCGCTCTCGC 3'). GoTaq PCR Core System II, which contains the components used in the PCR, was purchased from Promega Corporation (Madison, WI). The following PCR components were combined in a 0.5 mL microcentrifuge tube: 5.0 µL of 25 mM magnesium chloride solution, 10 µL of 5X green GoTaq flexi buffer, 1.0 µL of PCR nucleotide mix, containing 10 mM of each of the dNTPs, 1.5 µL of Fab1F primer, 1.5 µL of Fab1R primer, 0.5 µL of GoTaq polymerase, 20.5 µL of nuclease-free water, and 10 µL of DNA template from *Staphylococcus epidermidis*. Template DNA was genomic DNA prepared by boiling the cultures for five minutes. The PCR was allowed to occur in a thermocycler with the following conditions: 95 °C for three minutes, followed by 40 cycles of 95 °C for 45 seconds, 55 °C for one minute, and 72 °C for one minute. The final elongation cycle was allowed to occur at 72 °C for 10 minutes. PCR amplification of the *fab1* gene was conducted on both the

triclosan-exposed cells and the unexposed cells. PCR product was confirmed by using 1.5% agarose gel electrophoresis.

DNA Sequencing and Alignment

The PCR products were submitted to GeneWiz (South Plainfield, NJ) for direct sequencing by using the same primers as those used for the PCR. The resulting sequences were translated to peptide sequences by using EMBOSS Transeq Sequence Translation tools from EMBL-EBI. Both the DNA sequences and the peptide sequences were aligned by using EMBOSS Needle Pairwise Sequence Alignment tools from EMBL-EBI.

Kirby-Bauer Disc Diffusion Assay

The Kirby-Bauer disc diffusion assay was used to evaluate any differences in antibiotic resistance that might have occurred in both the unexposed and the triclosan exposed strains. The assay was conducted according to the manufacturer's instructions (BD, Franklin Lakes, NJ). Briefly, the bacterial strains were incubated in tubes containing TSB for 24 hours. The strains were then standardized in a spectrophotometer by using a 0.5 McFarland Standard at a wavelength of 595 nm. Bacterial lawns were then streaked onto Mueller-Hinton agar by using sterile cotton swabs so as to completely cover the Petri plates. Antibiotic-embedded filter paper discs were placed on the Petri plates by using an antibiotic disc dispenser. The antibiotics used were: ampicillin (10 µg), azithromycin (15 µg), ciprofloxacin (5 µg), gentamicin (10 µg), tetracycline (30 µg), and vancomycin (30 µg). Diameters of zones of inhibition were measured with a millimeter ruler.

Etest

The Etest was conducted on the triclosan-exposed and unexposed bacteria by following the instructions provided by the manufacturer (bioMérieux, Durham, NC). Briefly, the bacterial strains were incubated in tubes containing TSB for 24 hours. The strains were then standardized in a spectrophotometer by using a 0.5 McFarland Standard at a wavelength of 595 nm. Bacterial lawns were streaked onto Mueller Hinton agar, by using sterile cotton swabs. One E-strip was used per Petri plate and the results were read according to the Etest reading guide found in the Etest pack insert provided by the manufacturer. Antibiotics used in the Etest were the same as those used in the Kirby-Bauer disc diffusion assay.

Efflux Assay

A quantitative efflux-mediated multi-drug resistance assay was used according to Martins et. al., (2010) to determine whether subinhibitory triclosan exposure influenced the overexpression of efflux systems. Ethidium bromide (Sigma-Aldrich, St. Louis, MO) was prepared in distilled water at a stock concentration of 50 mg/L and was protected from light by storing it in bottles wrapped in aluminum foil. Tryptic soy agar plates containing the following concentrations of ethidium bromide were prepared: 0.0, 0.5, 1.0, 1.5, 2.0, and 2.5 mg/L. Twenty groups of cells, representing each of the five increases in triclosan MIC and their corresponding unexposed strains, were grown for 24 hours in 5.0 mL of TSB and standardized to an optical density of 0.6 at a 600 nm wavelength.

Pseudomonas aeruginosa (ATCC 24783) and *Escherichia coli* (ATCC 25922) were used as positive and negative controls, respectively, for efflux ability.

Bacterial samples were streaked with a sterile swab in a cartwheel pattern on tryptic soy agar (TSA) plates containing various concentrations of ethidium bromide. The plates were incubated for 18 hours, and observed under ultraviolet light and photographed. The minimal concentration of ethidium bromide that led to fluorescence was recorded.

RESULTS AND DISCUSSION

Effects of Triclosan Exposure

In this study, passage of *Staphylococcus epidermidis* SE1457 and SE1457Δica in subinhibitory concentrations of triclosan for 70 days resulted in an increase in the MIC of triclosan. For both strains, the initial triclosan MIC was 0.00015% and the final MIC was 1.5%. Hence, after 70 days of exposure to a 1/10-subinhibitory concentration of triclosan, the exposed cells became 10,000 times more resistant to triclosan than their corresponding unexposed strains (Fig. 3). The triclosan MIC increased at the same rate in both biofilm-positive and biofilm-negative strains. This suggested that the presence of an *ica* operon does not contribute to an increased ability to resist triclosan. The concentration of triclosan found in most personal care products is 0.15% meaning subinhibitory exposure resulted in resistance to the typical therapeutic dose of triclosan.

Chlorhexidine

The minimum inhibitory concentration of chlorhexidine was 0.00002% on all strains of *Staphylococcus epidermidis* used in this study. Thus, neither the ability to form a biofilm nor an increased ability to resist triclosan, regardless of extended exposure time, had any effect on the ability of the organism to resist chlorhexidine. These results further support the rationale that an increased resistance to chlorhexidine is unlikely due to the fact chlorhexidine is thought to have multiple targets (11).

DNA Sequencing and Alignment

One proposed mechanism of triclosan resistance in *Staphylococcus aureus* is changes in the permeability of the cell wall could prevent triclosan from reaching its target site (43). Other studies have shown that *fab1* mutation can lead to the development of triclosan resistance in organisms such as *Escherichia coli* and *Staphylococcus aureus* (16). It has also been demonstrated that a *fab1* mutation is required for triclosan resistance and that the altered *fab1* must be overexpressed at levels three- to fivefold higher than the level of expression in triclosan-sensitive strains (13).

In this study, sequencing of the *fab1* gene, amplified from the triclosan resistant *Staphylococcus epidermidis* SE1457 strain and the corresponding unexposed strain, showed a point mutation at position 235 in the triclosan exposed strain (Fig. 4). This mutation codes for an amino acid change from alanine to valine at position 95 in the protein sequence (Fig. 5). The Ala-95 in the unexposed strain has been shown to be part of the active site region of the Fab1-NAD^+-triclosan ternary complex (19). The 4-chloro substituent of triclosan accepts a hydrogen bond from the amide backbone of Ala-95 (19). These data suggested a *fab1* mutation could lead to the development of triclosan resistance in *Staphylococcus epidermidis* as a result of long-term subinhibitory triclosan exposure.

The difference between alanine and valine is that they contain a methyl side chain and an isopropyl side chain respectively. This indicates that the isopropyl side chain in valine blocks the ability of the 4-chloro substituent of triclosan from accepting the hydrogen bond from the amino acid backbone. This could interfere with the Fab1-NAD^+-

triclosan ternary complex, thereby preventing the triclosan from functioning to inhibit bacterial fatty acid elongation.

Kirby-Bauer Disc Diffusion Assay and Etest (Ampicillin)

In addition to triclosan exposure leading to an increased triclosan MIC, a series of Kirby-Bauer disc diffusion assays showed that cells, having evolved the ability to resist triclosan, also evolved an increased resistance to ampicillin whereas the unexposed strains did not. Etests confirmed the results of the Kirby-Bauer assays.

The ampicillin zone of inhibition increased from 25 mm to 30 mm in the unexposed SE1457 strain between 0 days and 70 days of passage (Fig. 6). The MIC increased from 0.016 μg/mL to 0.5 μg/mL (Fig. 7). The changes in the unexposed SE1457 strain was most likely due to a random error in the standardization of the cells. The ampicillin zone of inhibition decreased in diameter from 25 mm to 6 mm in the triclosan exposed SE1457 strain between 0 days and 70 days of triclosan exposure (Fig. 6). The MIC increased from 0.016 μg/mL to 1.0 μg/mL (Fig. 7). According to the Zone Diameter Interpretive Chart from BD, staphylococci are considered to be resistant to ampicillin if the zone diameter around the ampicillin impregnated disc is ≤28 mm; hence, the triclosan-exposed SE1457 strains with triclosan MICs of 0.15% and 1.5% both evolved resistance to ampicillin.

The ampicillin zone of inhibition increased in diameter from 25 mm to 30 mm in the unexposed SE1457Δica strain between 0 days and 70 days of passage (Fig. 6). The MIC increased from 0.016 μg/mL to 0.023 μg/mL (Fig. 7). These changes were most likely due to a random error in the standardization of the cells. The ampicillin zone of

inhibition decreased in diameter from 25 mm to 6 mm in the triclosan exposed SE1457Δica strain between 0 days and 70 days of subinhibitory triclosan exposure (Fig. 6). The MIC increased from 0.016 μg/mL to 0.75 μg/mL (Fig. 7). Hence the triclosan exposed SE1457Δica strains with triclosan MICs of 0.15% and 1.5% both evolved resistance to ampicillin.

Ampicillin is known to interfere with cell wall synthesis by binding to penicillin-binding proteins inside the cell wall (34). A recent study showed that the exposure of *Staphylococcus aureus* to sublethal concentrations of penicillin caused two cell wall proteins to shift from the peripheral wall to the septum, which was most likely due to an antibiotic mediated increase of free anchoring sites at the septum (52). In a similar manner, it is possible triclosan exposure could caused the shifting of the penicillin-binding proteins, therefore accounting for this ampicillin resistance.

Kirby-Bauer Disc Diffusion Assay and Etest (Tetracycline)

In addition to triclosan exposure leading to an increased triclosan MIC and ampicillin resistance, a series of Kirby-Bauer disc diffusion assays showed that cells, having evolved the ability to resist triclosan, also evolved an increased resistance to tetracycline whereas the unexposed strains did not. Etests confirmed the results of the Kirby-Bauer assays.

The tetracycline zone of inhibition decreased in diameter from 30 mm to 26 mm in the unexposed SE1457 strain between 0 days and 70 days of passage (Fig. 8). The MIC decreased from 0.5 μg/mL to 0.094 μg/mL (Fig. 9). These changes were most likely due to random error in the standardization of the cells. The tetracycline zone of inhibition

decreased in diameter from 30 mm to 10 mm in the triclosan exposed SE1457 strain between 0 days and 70 days of subinhibitory triclosan exposure (Fig. 8). The MIC increased from 0.5 μg/mL to 32 μg/mL (Fig. 9). According to the Zone Diameter Chart from BD, staphylococci are considered to be resistant to tetracycline if the zone diameter around the tetracycline impregnated disc is ≤14 mm, hence the triclosan exposed SE1457 strains with triclosan MICs of 0.15% and 1.5% both evolved resistance to tetracycline.

There were no changes in the tetracycline zones of inhibition in the unexposed SE1457Δica strain between 0 days and 70 days of passage (Fig. 8). The MIC decreased from 0.5 μg/mL to 0.125 μg/mL (Fig. 9). This change was most likely due to random error in the standardization of the cells. The tetracycline zone of inhibition decreased in diameter from 30 mm to 7 mm in the triclosan exposed SE1457Δica strain between 0 days and 70 days of subinhibitory triclosan exposure (Fig. 8). The MIC increased from 0.5 μg/mL to 96 μg/mL (Fig. 9). Hence, the triclosan exposed SE1457Δica strains with triclosan MICs of 0.15% and 1.5% both evolved resistance to tetracycline.

Tetracyclines inhibit the synthesis of protein by binding to the 30S ribosomal subunit and blocking the attachment of aminoacyl-tRNA to the acceptor site of the mRNA ribosome complex, thus preventing the introduction of new amino acids to the nascent polypeptide chain (1).

Two tetracycline resistance mechanisms have been identified in staphylococci. They are the acquisition plasmids carrying *tetK* and *tetL* genes, which result in active efflux, and *tetM* or *tetO* determinants carried on either the chromosome or transposons, which mediate ribosomal protection (41). MGE mediated resistance is unlikely due to the fact these experiments were carried out in pure culture in a closed system. A more likely

resistance mechanism is the production of ribosomal protection proteins due to a chromosomal mutation. Possibly the cell wall has been altered in a way that has decreased its permeability.

Kirby-Bauer Assay and Etest (Azithromycin, Gentamicin, and Vancomycin)

With respect to resistance, there was no difference in the zones of inhibition or MICs of azithromycin, gentamicin, or vancomycin on either the triclosan exposed cells or the unexposed cells (Fig. 10 to 13). These data suggest long-term exposure to subinhibitory triclosan does not influence an increased resistance to these antibiotics.

Kirby-Bauer Disc Diffusion Assay and Etest (Ciprofloxacin)

The unexposed strain of SE1457Δica displayed an increased resistance to ciprofloxacin after being passed in TSB for 70 days. The results of a Kirby-Bauer disc diffusion assay showed a decrease in the diameter of zone of inhibition around the disc impregnated with ciprofloxacin from 30 mm to 15 mm (Fig. 10). The MIC increased from 0.064 μg/mL to 3.0 μg/mL (Fig. 11). According to the Zone Diameter Interpretive Chart from BD, staphylococci are considered to be resistant to ciprofloxacin if the zone diameter around the ciprofloxacin impregnated disc is ≤15 mm, hence the unexposed SE1457Δica strain of *Staphylococcus epidermidis* evolved resistance to ciprofloxacin in the absence of selection pressure. This is likely due to a copying error during DNA replication. None of the other strains exhibited resistance to ciprofloxacin.

There are two broad mechanisms of fluoroquinolone resistance, which occur as a result of chromosomal mutation (20). The mechanisms are alterations that limit the

permeation of the drug to the target and alterations in the target enzymes of the drug (20). In Gram-positive organisms, the target enzyme is topoisomerase IV (35). Although plasmid-mediated ciprofloxacin resistance has been observed, the data presented in this thesis suggested a chromosomal mutation was the most likely mechanism for resistance since the strains in this study were grown in pure culture. The data also shed light on the inherent genetic flexibility of *Staphylococcus epidermidis*.

Efflux Assay

The efflux-assay uses ethidium bromide, which is a universal efflux pump substrate (30). Ethidium bromide functions by binding with DNA and intercalating between its hydrophobic base pairs. This intercalation causes the DNA to stretch, removing water molecules from the ethidium cation. The resulting distortion of the double helix interferes with DNA replication, transcription, and DNA repair. This dehydration resulted in an increased fluorescence of the ethidium and the cell. The assay is based on the rationale that there is a maximum ethidium bromide concentration that can be effectively extruded by cells (30). Any concentration greater than this maximum will be retained by the cell and will lead to the detection of fluorescence when exposed to ultraviolet light (30). The smallest concentration of ethidium bromide that leads to fluorescence is the highest concentration of ethidium bromide that the bacteria can exclude (30).

In addition to providing a method of ranking bacterial strains according to efflux capability, this assay also allows for the observation of ethidium bromide resistance.

In this study, the assays showed no evidence of an increased efflux capability and the bacterial strains were, therefore, not quantitatively ranked. The assay did, however, show a correlation between increased triclosan MIC and the ability of *Staphylococcus epidermidis* to grow in increasing concentrations of ethidium bromide (Fig. 14 to 17).

All of the unexposed SE1457 strains were inhibited by 2.0 mg/L of ethidium bromide (Fig. 14). The triclosan exposed SE1457 strains, having been exposed to subinhibitory concentrations of triclosan for 56 days and 70 days, grew in 2.5 mg/L of ethidium bromide (Fig. 15). All of the unexposed SE1457Δica strains were inhibited by 2.0 mg/L of ethidium bromide (Fig. 16). The triclosan exposed strains of SE1457Δica, having been exposed to subinhibitory triclosan concentrations for 42 days, 56 days, and 70 days, grew in 2.5 mg/L of ethidium bromide (Fig. 17). All of the strains that grew in 2.5 mg/L of ethidium bromide demonstrated growth in 4.0 mg/L of ethidium bromide (figures not shown).

At physiological ionic strength, ethidium is very sensitive to the composition and sequence of polymeric nucleic acids (26). Ethidium has a 100-fold higher affinity to poly d(AT)-poly d(AT) as compared to poly d(A)-poly d(T) (26). It also exhibits a preference for the alternating purine-pyrimidine tract of poly d(GC)-poly d(CG) as compared to poly d(G)-poly d(C) (26). Additionally, ethidium exhibits a 10-fold higher affinity to poly d(G)-d(C) over poly d(A)-d(T) (26). Luedtke et. al., (2003) and the data from this thesis suggest there might have been chromosomal mutations profound enough to lower the binding affinity of ethidium bromide to the DNA of the bacterial strains exposed to higher concentrations of triclosan, thereby decreasing the susceptibility of those strains to the ethidium bromide.

CONCLUSIONS

A study conducted in 1990 in which 12 populations of *Escherichia coli* were allowed to evolve for 2,000 generations showed an increase of about 37% in mean fitness (25). Eighteen thousand generations later, two of those populations were examined for the parallel evolution of gene-expression profiles when compared to the original ancestor population (8). The expression of 59 genes changed significantly in both populations in the same direction relative to the ancestor (8). This profusion of change, despite the lack of selection pressure, substantiates the rationale that selection pressure might lead to a pattern of parallel evolution even more expeditious than demonstrated in this study.

The fact that two triclosan-resistant strains of *Staphylococcus epidermidis* exhibited resistance to ampicillin and tetracycline as well as a decreased susceptibility to ethidium bromide, despite each of these antibacterial agents having different modes of action, could be indicative of several phenomena.

One potential phenomenon is a mechanism leading to an increased cell wall thickness relative to increased selection pressure. This phenomenon has been observed in association with vancomycin resistance in *Staphylococcus epidermidis* (14). Another potential explanation is triclosan exposure caused diminished cell wall permeability, which could be the result of multiple factors, including the shifting of cell wall proteins, which has been shown to occur in *Staphylococcus aureus* in association with sublethal ampicillin exposure (52). Mutation in the *fabI* gene was most likely the cause of triclosan resistance, and some other chromosomal mutation is most likely the cause of resistance to

the other antibacterial agents since all of the experiments in this study were carried out in pure culture.

This demonstration of a 10,000-fold increase in triclosan resistance in *Staphylococcus epidermidis* over 70 days, due to the application of selection pressure, demonstrates the antimicrobial resistance problem associated with the overuse and misuse of antibacterial agents. The results also provide evidence that distribution of over-the-counter antimicrobials into the environment can induce resistance to that particular antimicrobial in addition to certain clinical antibiotics. This thesis supported the rationale that triclosan as well as other disinfectants should only be used circumspectly where clear health benefits can be discerned (24).

Further studies should continue to investigate, identify, and understand other potential antibiotic resistance mechanisms. It is also necessary to understand the link between triclosan resistance and this newly acquired multi-drug resistance phenotype. Several proposed resistance mechanisms have been discussed; however, it is also possible that resistances mechanisms that have not yet been reported are the causes of this multi-drug resistance phenotype.

The relatively recent antibiotic-as-beneficial-signal hypothesis suggests antibiotics in nature evolved as a communication method between unrelated microbial species, but, if introduced to a bacterial population at a high enough concentration, can cause death (40). Work in the lab of Julian Davies over the last 15 years has indicated antibiotics made by microbes perform multiple functions and that the molecules are more often a means of communication than of inhibition (32).

The microbial detection of low concentrations of antibiotics might be interpreted as a warning for future increased concentrations, which could allow the organism to respond in a manner that reduces susceptibility (40). *Pseudomonas aeruginosa*, for example, forms a biofilm as a response to subinhibitory tetracycline concentrations, thereby reducing its exposure to future antibiotics (40). This study shows *Staphylococcus epidermidis* has a similar inherent ability to respond to triclosan, thereby initiating the observed change in the Fab1 sequence.

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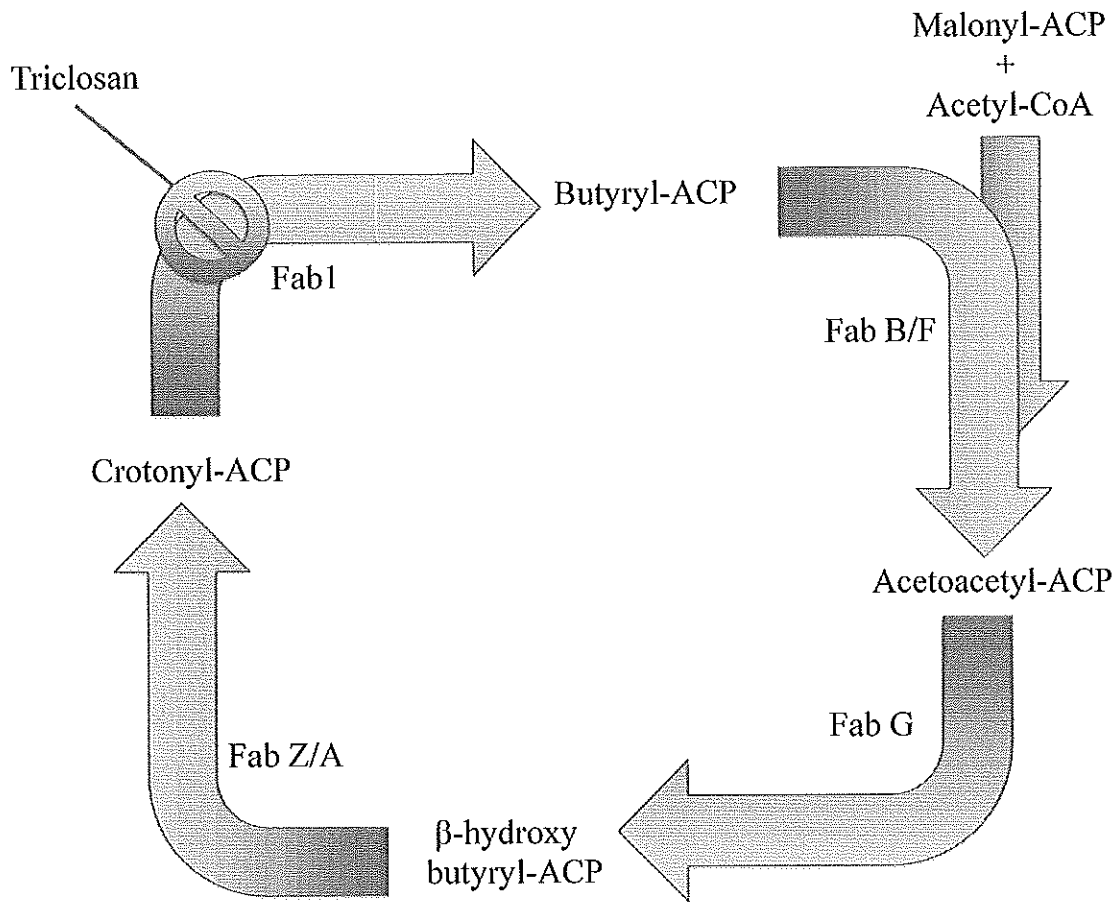

FIG. 1. The triclosan mode of action is to target enoyl-acyl carrier protein reductase (Fab1), the final enzyme in the fatty acid elongation cycle, by using NADH to reduce the double bond of Fab1 (Adapted from Patel et. al., 2008).

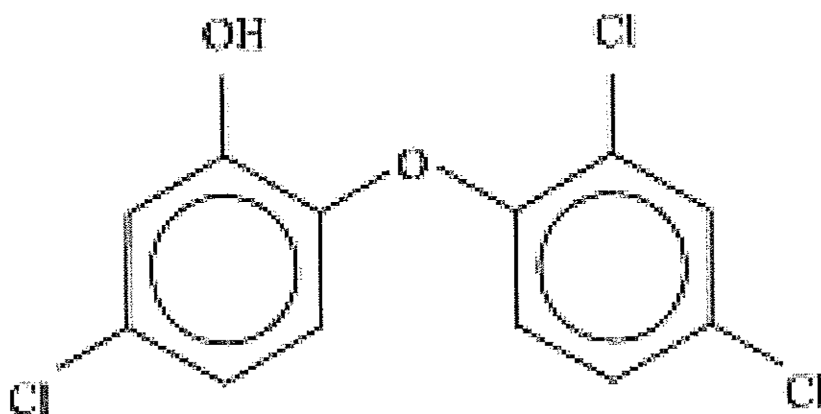

FIG. 2. Molecular structure of 2,4,4'-trichloro-2'-hydroxydiphenyl ether (triclosan) (From Margaretha et. al., 2001).

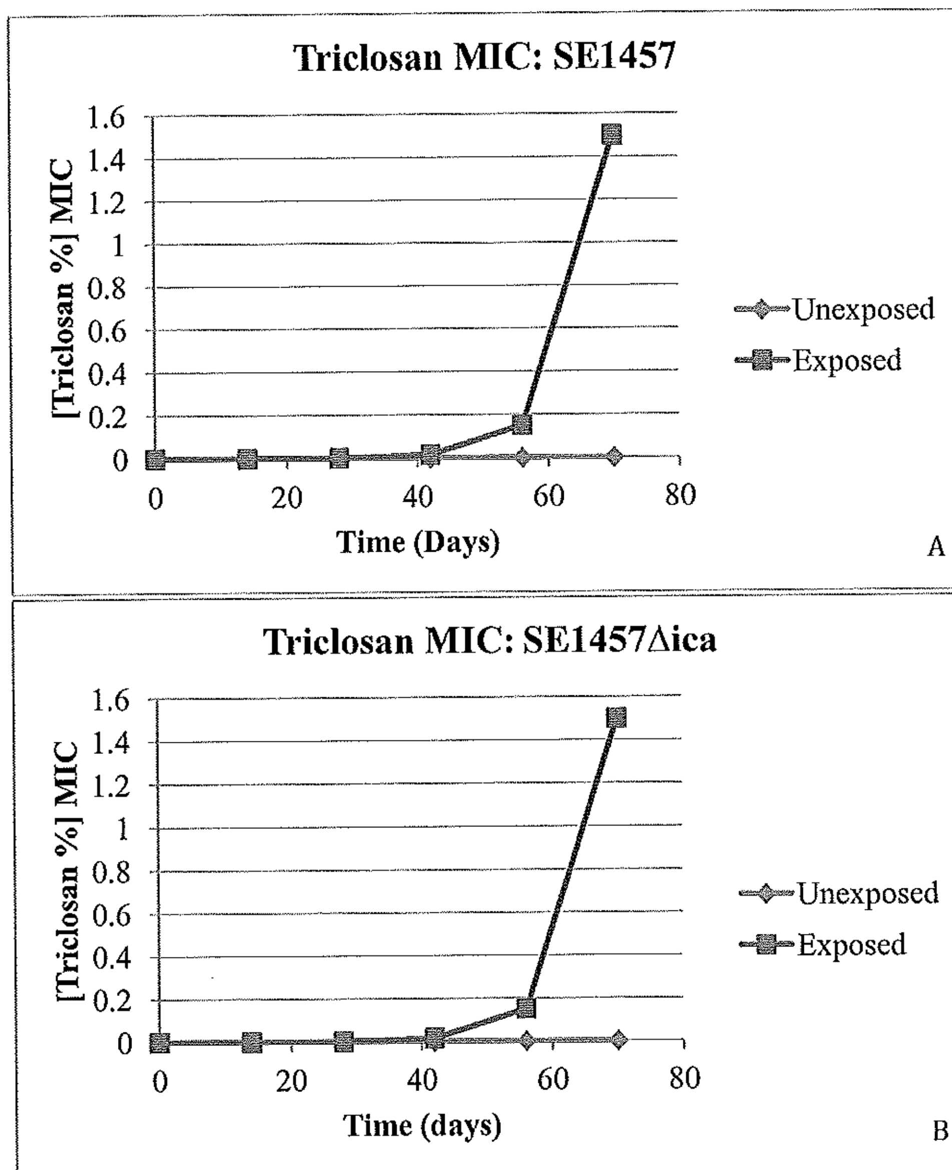

FIG. 3. MIC of triclosan on (A) unexposed SE1457 compared to triclosan-exposed SE1457, and (B) unexposed SE1457Δica compared to triclosan-exposed SE1457Δica.

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Query     94 TAGAGAAATTATTAGAACAATTAAATCAATCTGAACATCATCTCTATGAA    143
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Query    194 AAAAGATGTAGGCCAGATTGATGGTGTTTATCACTCAATCGTATTTGCCA    243
             |||||||||||||||||||||||||||||||||||||||||.||||||||
Sbjct    501 AAAAGATGTAGGCCAGATTGATGGTGTTTATCACTCAATCGCATTTGCCA    550

Query    244 ATATGGAAGATTTACGAGGTCGATTTTCAGAAACATCTCGCGAAGGTTTC    293
             ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct    551 ATATGGAAGATTTACGAGGTCGATTTTCAGAAACATCTCGCGAAGGTTTC    600

Query    294 TTACTTGCACAAGAAATTAGTTCATATTCACTTACTCTCGTAGCTCATGA    343
             ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct    601 TTACTTGCACAAGAAATTAGTTCATATTCACTTACTCTCGTAGCTCATGA    650

Query    344 AGCCAAAAAACTTATGCCTGAAGGTGGAAGTATTGTTGCGACGACTTATA    393
             ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct    651 AGCCAAAAAACTTATGCCTGAAGGTGGAAGTATTGTTGCGACGACTTATA    700

Query    394 TTGGTGGTGAGGCAGCAGTTCAAAACTATAATGTTATGGGTGTAGCTAAA    443
             ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct    701 TTGGTGGTGAGGCAGCAGTTCAAAACTATAATGTTATGGGTGTAGCTAAA    750

Query    444 GCAAGTTTAGAGGCGAATGTTAAATATTTAGCTTTAGACTTAGGTGAAGA    493
             ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct    751 GCAAGTTTAGAGGCGAATGTTAAATATTTAGCTTTAGACTTAGGTGAAGA    800

Query    494 TAATATTCGTGTCAATGCTATTTCTGCAGGGCCAATTCGTACTTTAAGTG    543
             ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct    801 TAATATTCGTGTCAATGCTATTTCTGCAGGGCCAATTCGTACTTTAAGTG    850
```

FIG. 4. Nucleotide alignment from EMBOSS for SE1457 *fab1* from the unexposed strain (Sbjct) and triclosan-exposed strain after 70 days of exposure (Query) generated from the forward and reverse primers. The point mutation of position 235 is indicated by a circle.

```
Query   18  GVAKVLDRLGAKLVFTYRKERSRKELEKLLEQLNQSEHHLYEIDVQNDED   197
            ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   23  GVAKVLDRLGAKLVFTYRKERSRKELEKLLEQLNQSEHHLYEIDVQNDED   82

Query   198 IINGFSQIGKDVGQIDGVYHSIVFANMEDLRGRFSETSREGFLLAQEISS   377
            ||||||||||||||||||||||.|||||||||||||||||||||||||||
Sbjct   83  IINGFSQIGKDVGQIDGVYHSIAFANMEDLRGRFSETSREGFLLAQEISS   142

Query   378 YSLTLVAHEAKKLMPEGGSIVATTYIGGEAAVQNYNVMGVAKASLEANVK   557
            ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   143 YSLTLVAHEAKKLMPEGGSIVATTYIGGEAAVQNYNVMGVAKASLEANVK   202

Query   558 YLALDLGEDNIRVNAISAGPIRTLSAKGVGGFNTILKEIEARAP   608
            ||||||||||||||||||||||||||||||||||||||||||||
Sbjct   203 YLALDLGEDNIRVNAISAGPIRTLSAKGVGGFNTILKEIEARAP   219
```

FIG. 5. Partial protein alignment results from blastx for SE1457 Fab1 sequences from the unexposed strain (Sbjct) and triclosan-exposed strain after 70 days of exposure (Query). The mutation of alanine (A) to valine (V) at position 95 is indicated by a circle.

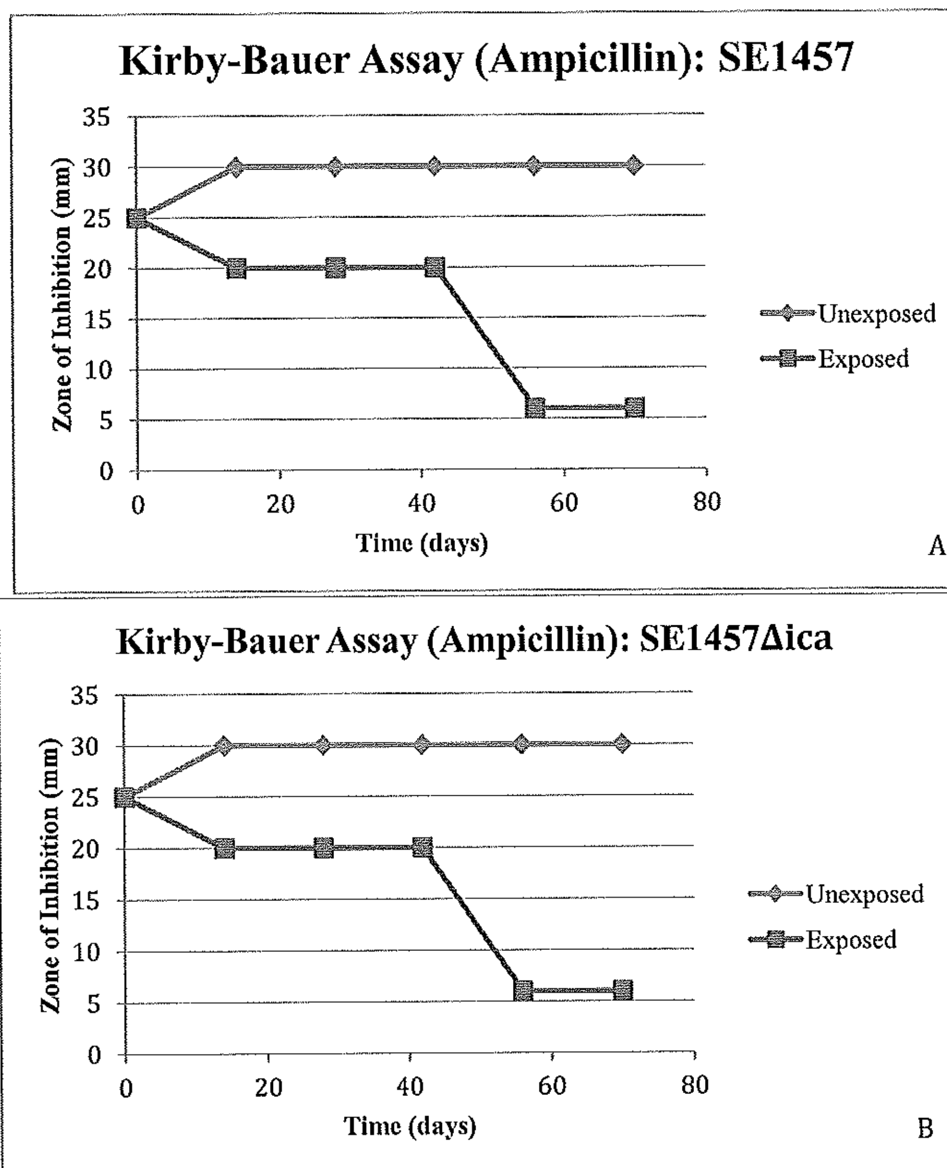


FIG. 6. Ampicillin zone of inhibition of (A) unexposed SE1457 compared to triclosan-exposed SE1457, and (B) unexposed SE1457Δica compared to triclosan-exposed SE1457Δica.

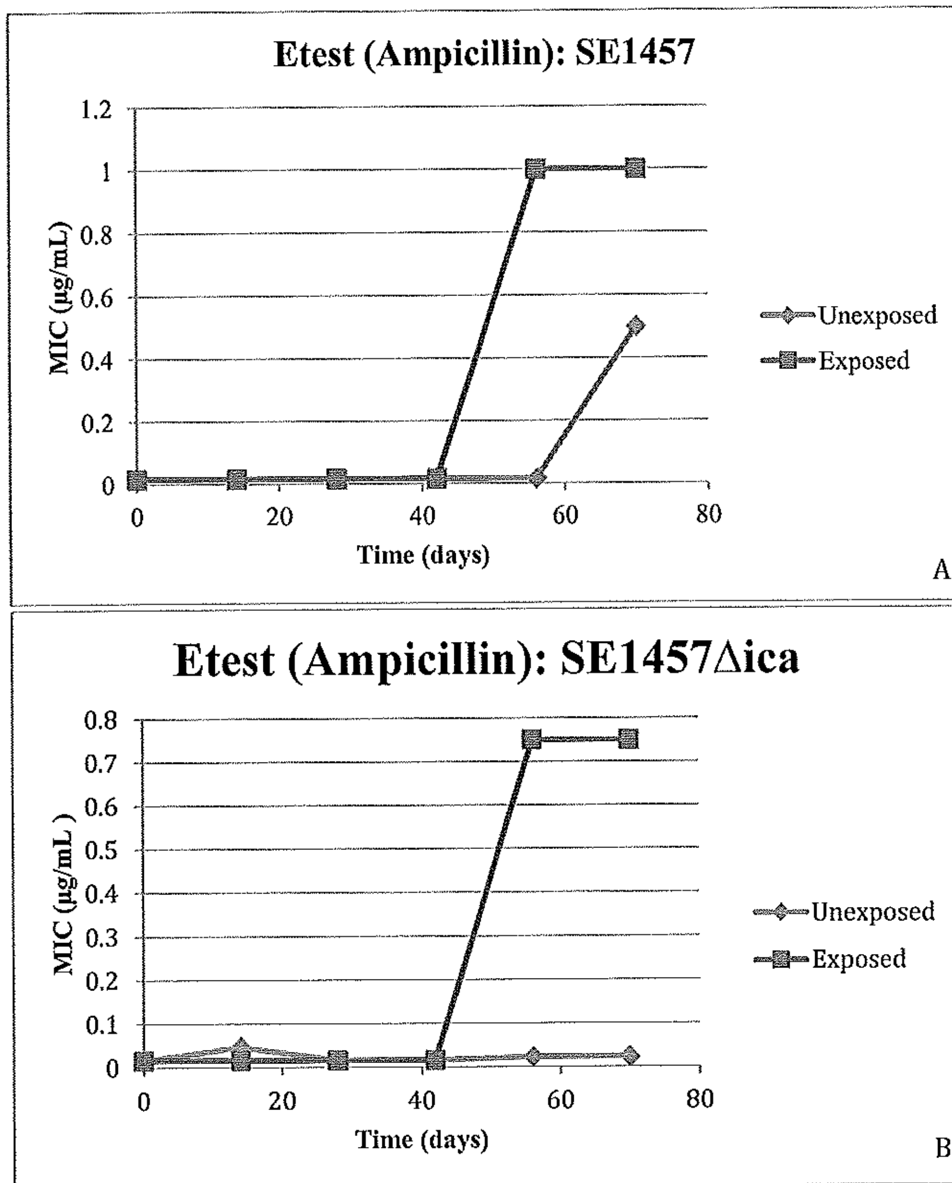


FIG. 7. MIC of ampicillin on (A) unexposed SE1457 compared to triclosan-exposed SE1457, and (B) unexposed SE1457Δica compared to triclosan-exposed SE1457Δica as determine by Etests.

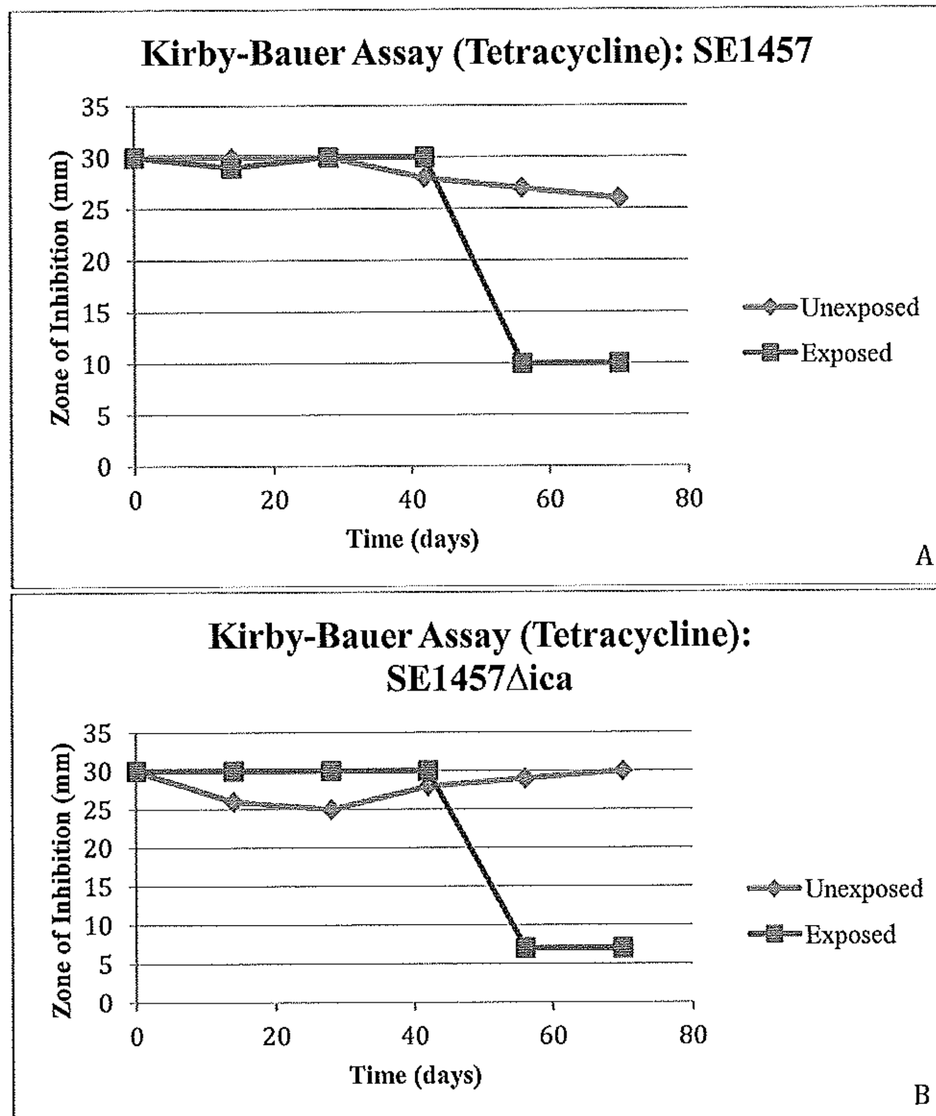


FIG. 8. Tetracycline zone of inhibition of (A) unexposed SE1457 compared to triclosan-exposed SE1457, and (B) unexposed SE1457Δica compared to triclosan-exposed SE1457Δica.

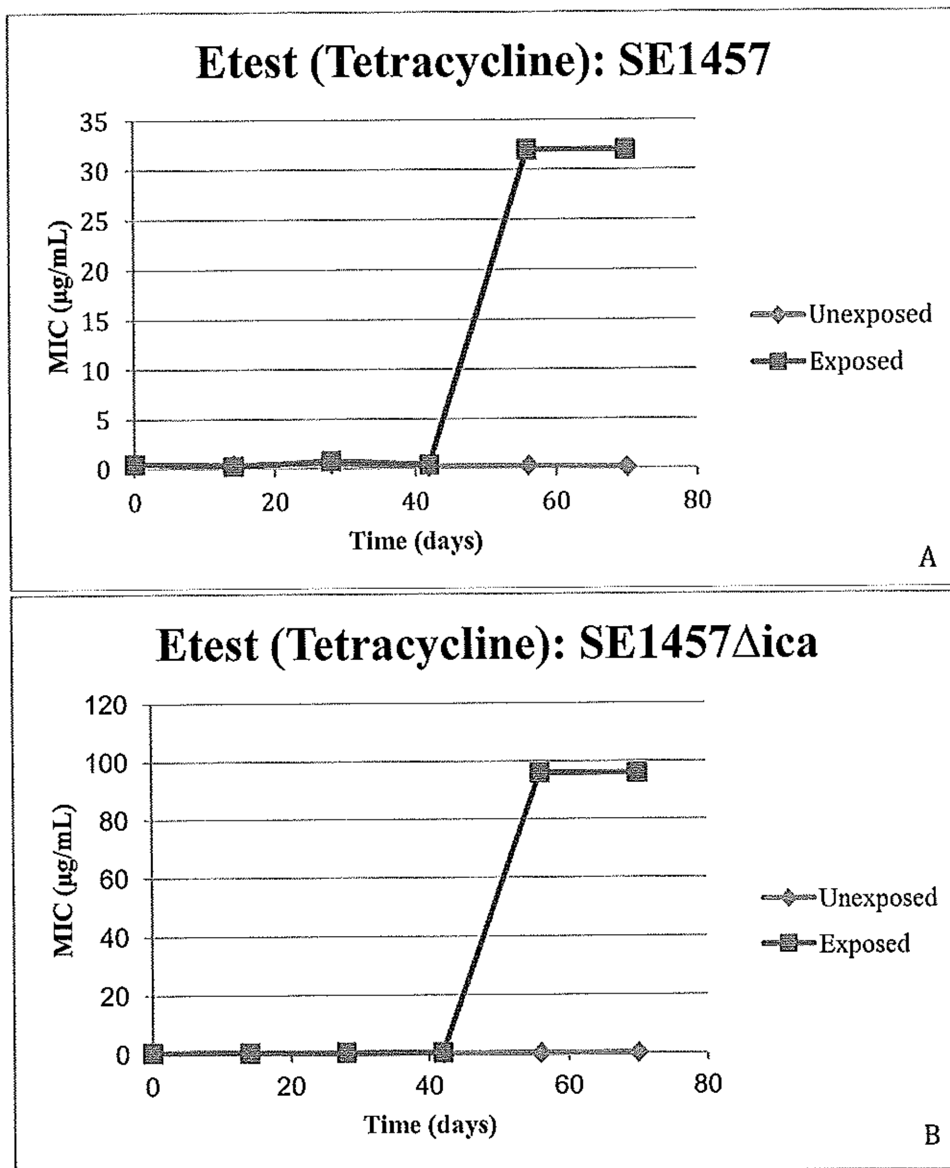


FIG. 9. MIC of tetracycline on (A) unexposed SE1457 compared to triclosan-exposed SE1457, and (B) Unexposed SE1457Δica compared to triclosan-exposed SE1457Δica as determined by Etests.

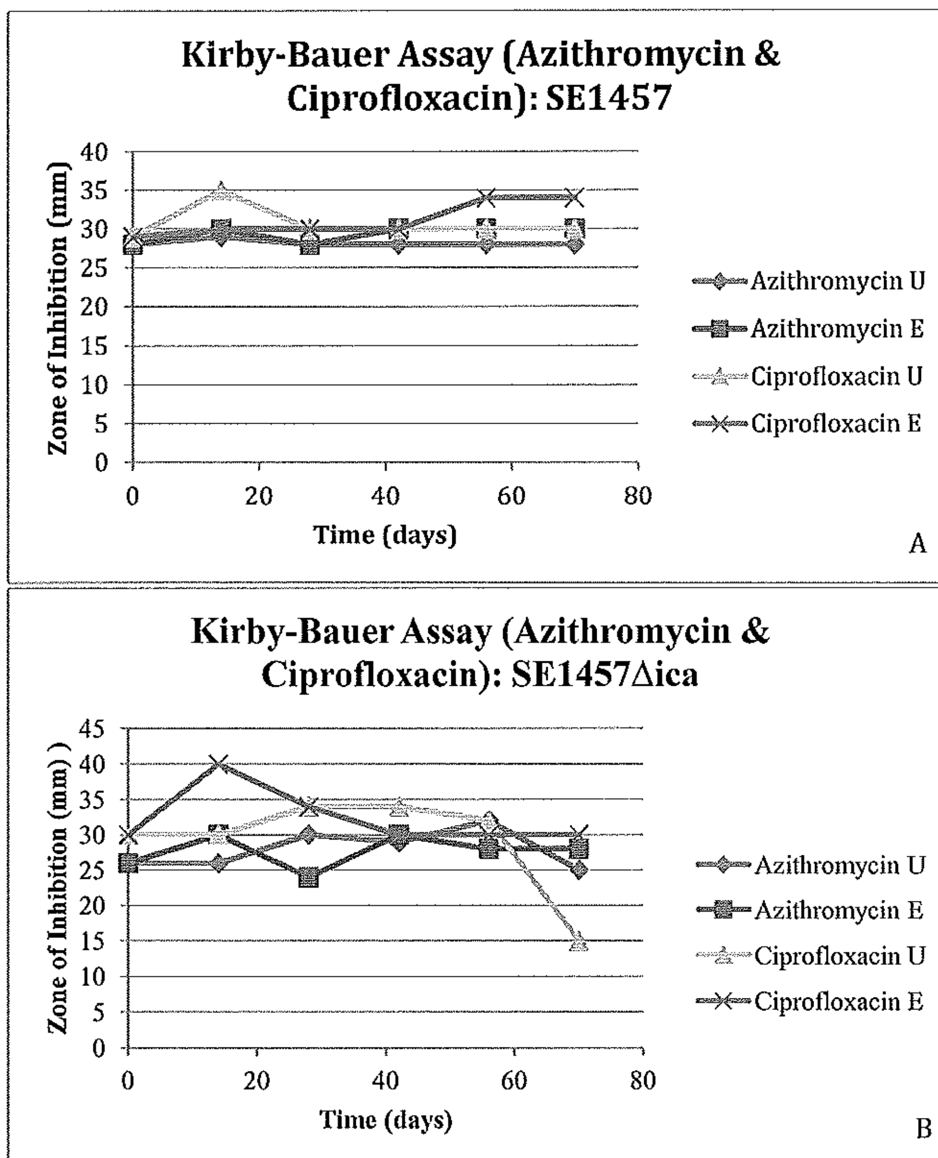


FIG. 10. Azithromycin and ciprofloxacin zones of inhibition of (A) unexposed SE1457 compared to triclosan-exposed SE1457, and (B) unexposed SE1457Δica compared to triclosan-exposed SE1457Δica.

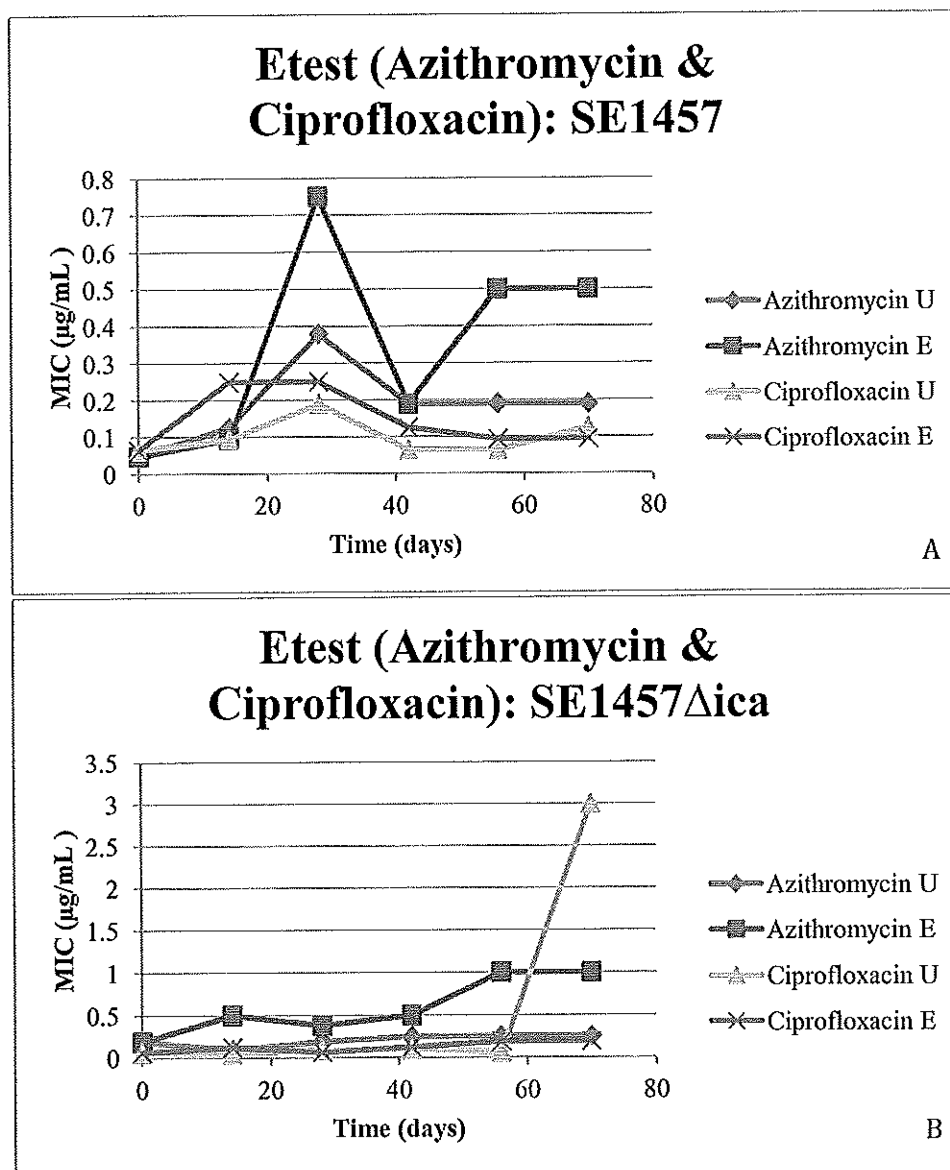


FIG. 11. MIC of azithromycin and ciprofloxacin on (A) unexposed SE1457 compared to triclosan-exposed SE1457, and (B) unexposed SE1457Δica compared to triclosan-exposed SE1457Δica as determined by Etests.

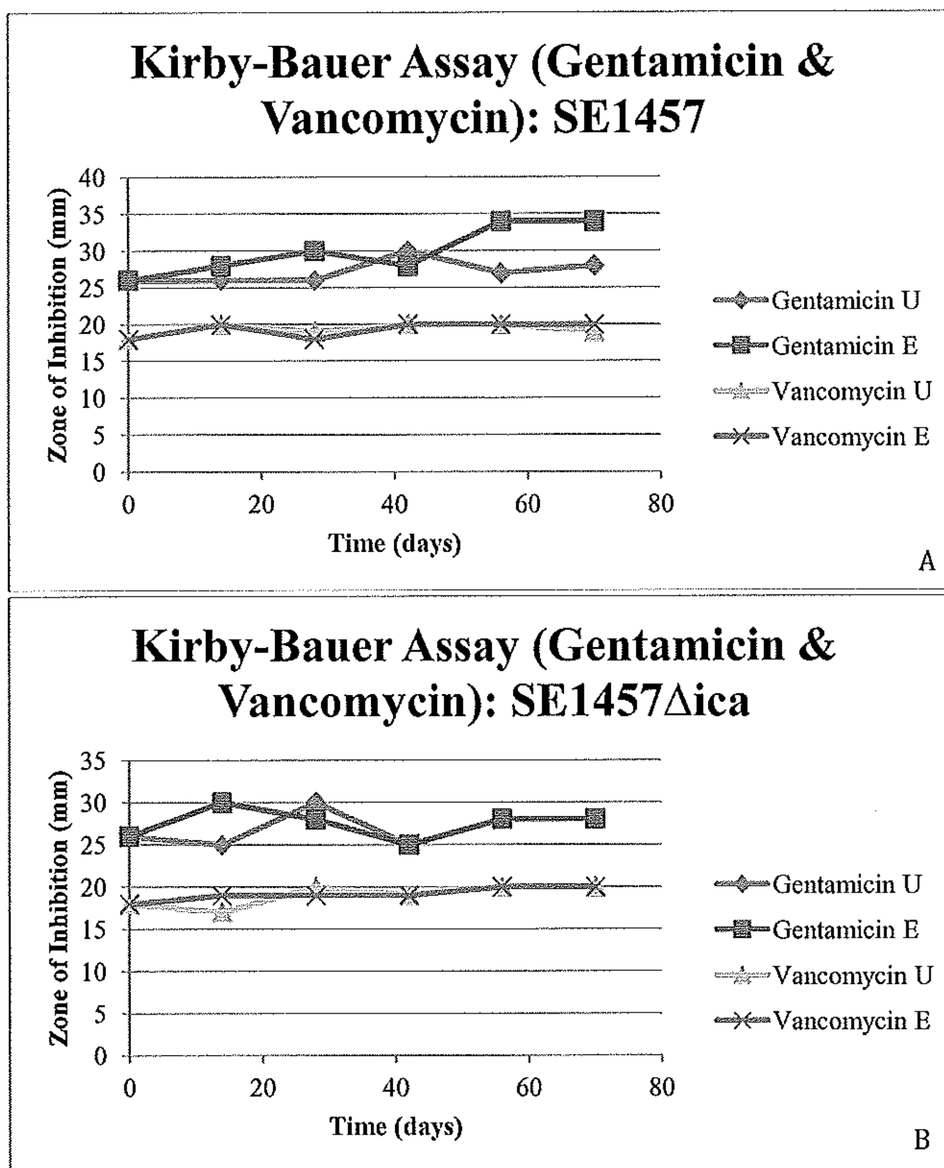


FIG. 12. Gentamicin and vancomycin zones of inhibition of (A) unexposed SE1457 compared to triclosan-exposed SE1457, and (B) unexposed SE1457Δica compared to triclosan-exposed SE1457Δica.

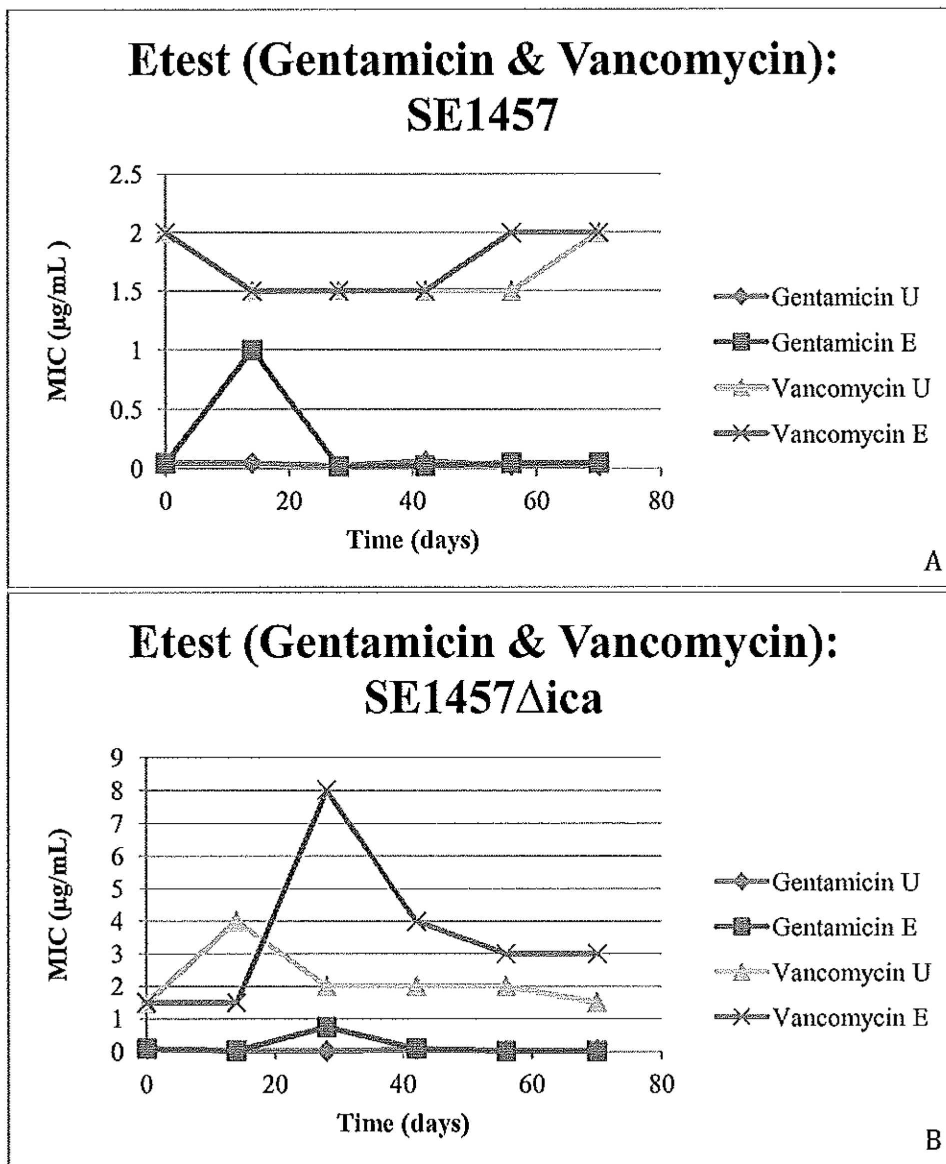


FIG. 13. MIC of gentamicin and vancomycin on (A) unexposed SE1457 compared to triclosan-exposed SE1457, and (B) unexposed SE1457Δica compared to triclosan-exposed SE1457Δica as determined by Etests.

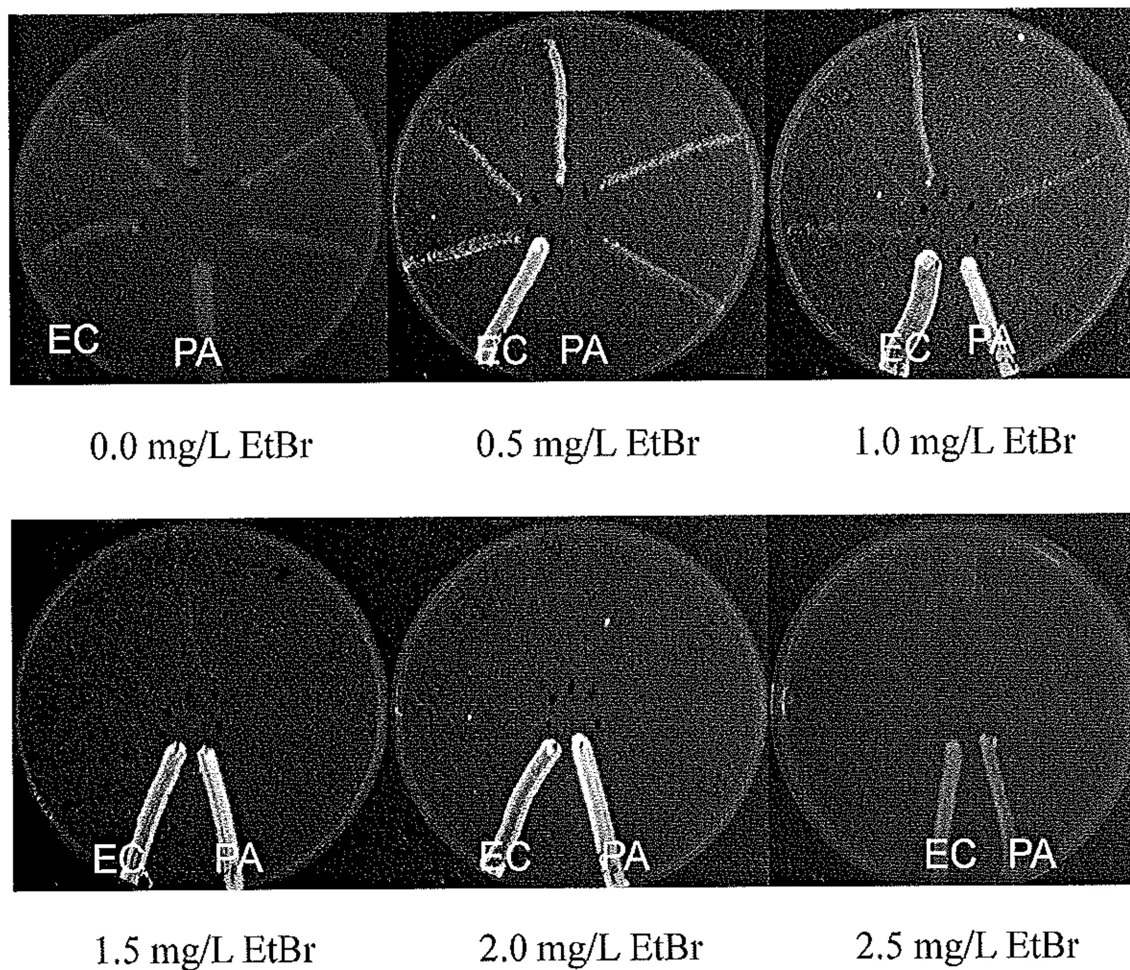


FIG. 14. Evaluation of efflux activity of unexposed SE1457 strains. In a counterclockwise fashion, the strains are *Pseudomonas aeruginosa* (PA), *Staphylococcus epidermidis* passed in TSB for 14, 28, 42, 56, and 70 days, and *Escherichia coli* (EC).

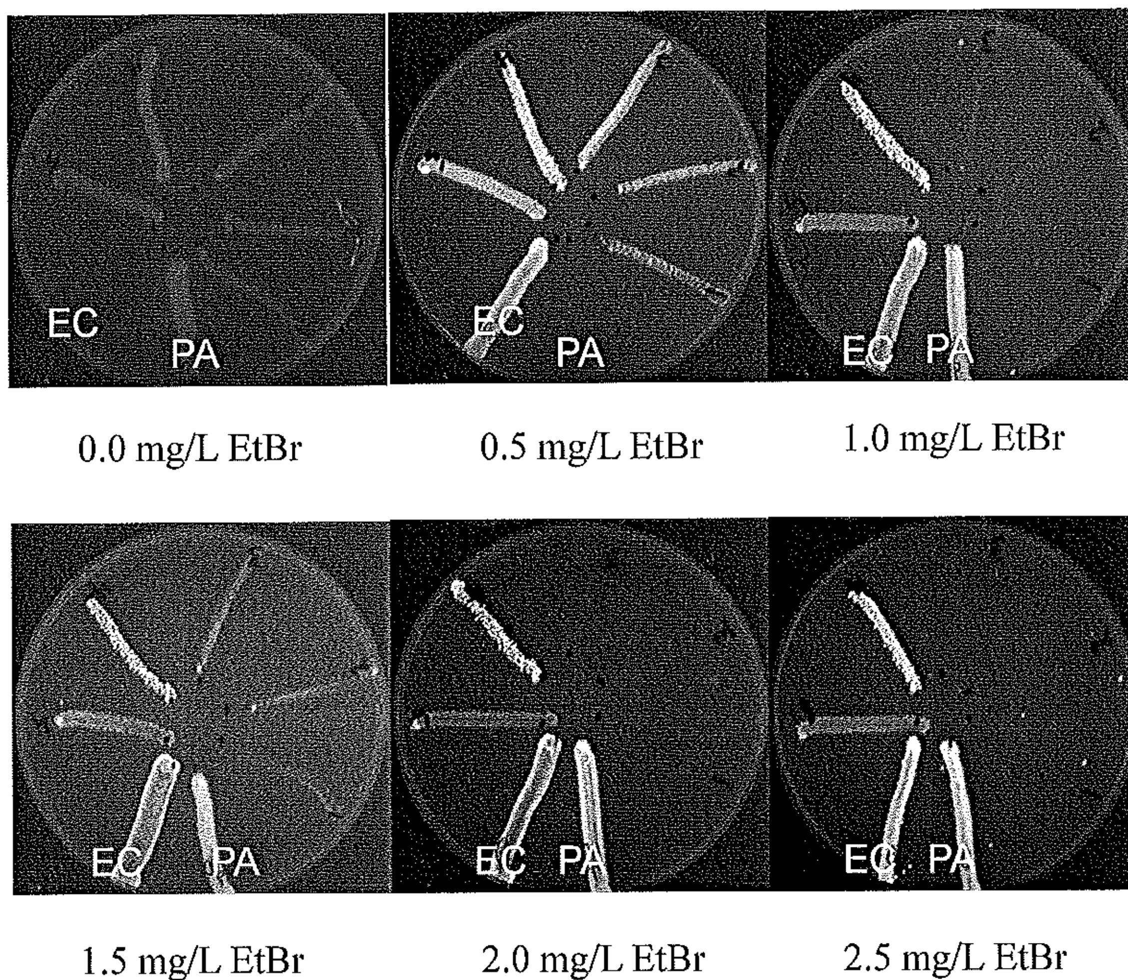


FIG. 15. Evaluation of efflux activity of triclosan-exposed SE1457 strains. In a counterclockwise fashion, the strains are *Pseudomonas aeruginosa* (PA), *Staphylococcus epidermidis* exposed to subinhibitory triclosan for 14, 28, 42, 56, and 70 days, and *Escherichia coli* (EC).

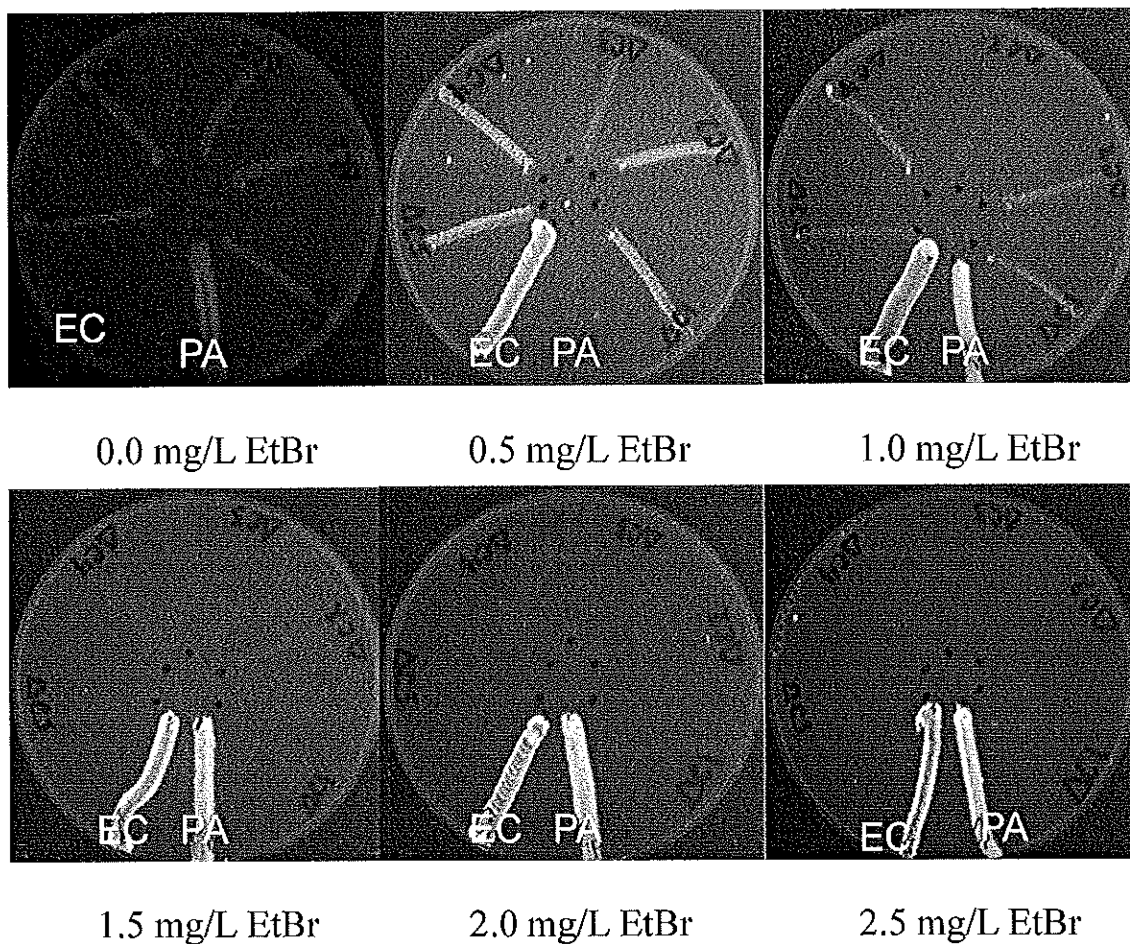


FIG. 16. Evaluation of efflux activity of unexposed SE1457Δica. In a counterclockwise fashion, the strains are *Pseudomonas aeruginosa* (PA), *Staphylococcus epidermidis* passed in TSB for 14, 28, 42, 56, and 70 days, and *Escherichia coli* (EC).

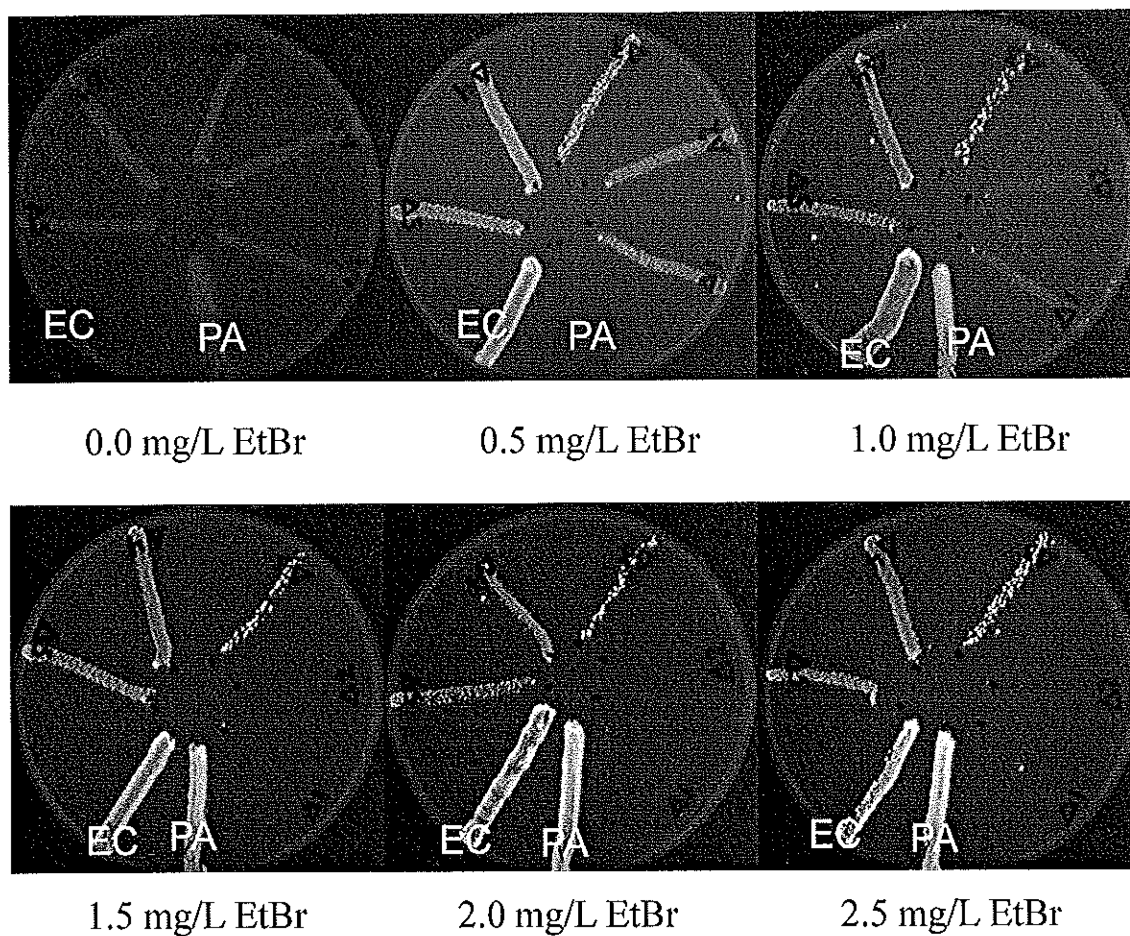
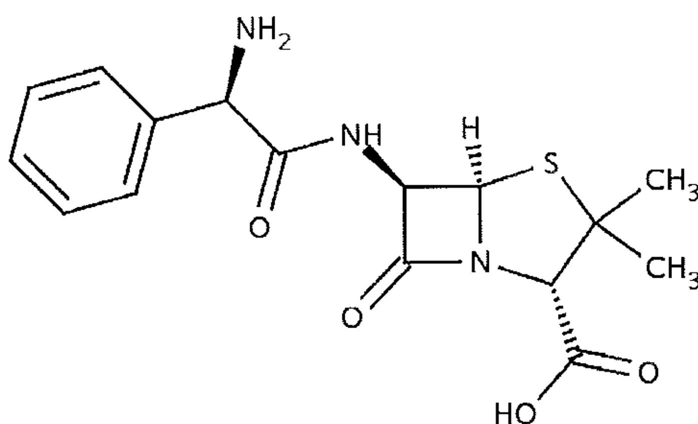


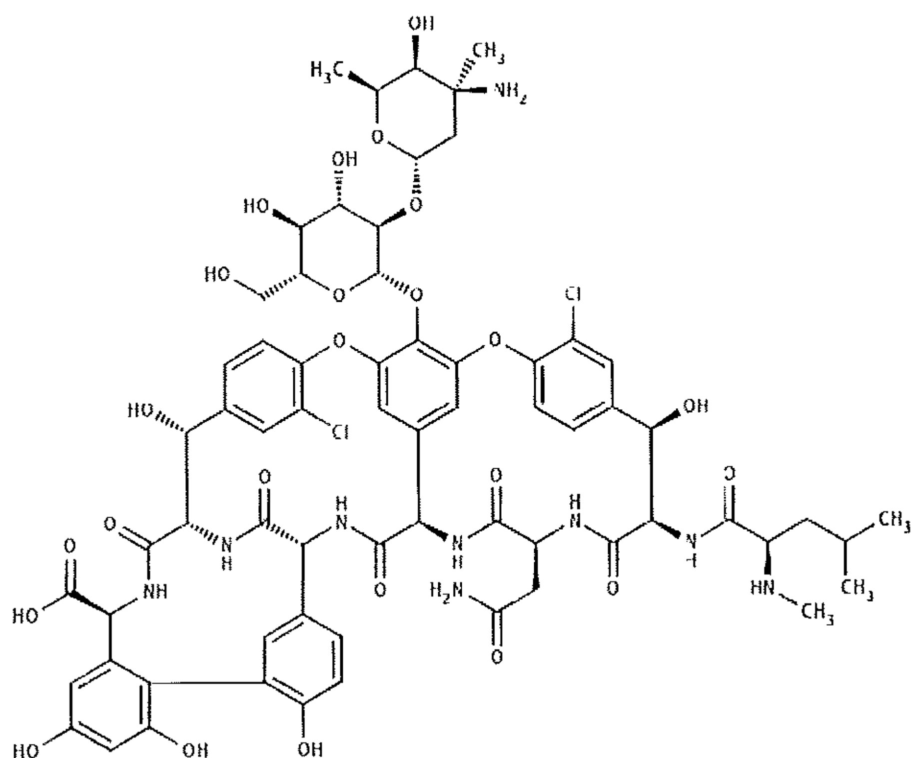
FIG. 17. Evaluation of efflux activity of triclosan-exposed SE1457Δica strains. In a counterclockwise fashion, the strains are *Pseudomonas aeruginosa* (PA), *Staphylococcus epidermidis* exposed to subinhibitory triclosan concentrations for 14, 28, 42, 56, and 70 days, and *Escherichia coli* (EC).

Appendix A: Molecular structures of the cell wall inhibitor antibiotics, (A) ampicillin, which is a semisynthetic penicillin thought to function by inhibiting the final step in bacterial cell wall synthesis leading to the lyses of the cell, and (B) vancomycin, which is a glycopeptide that also inhibits cell wall synthesis (1, 11).

A

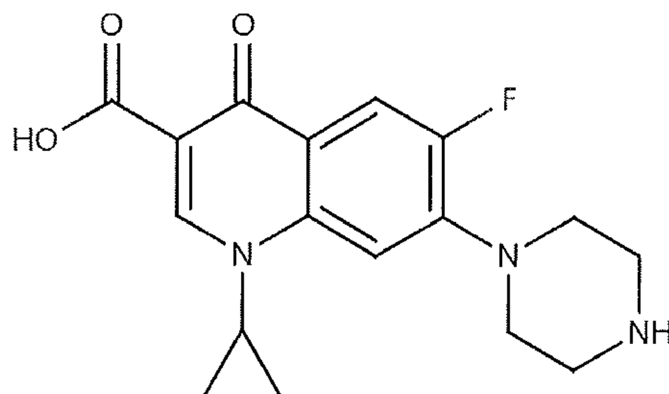


B

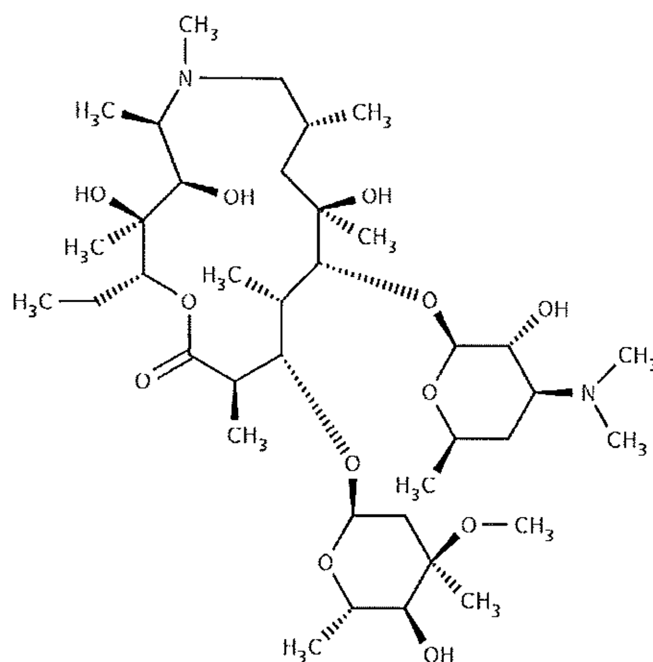


Appendix B: Molecular structures of (A) ciprofloxacin, which is a fluoroquinolone that inhibits bacterial nucleic acid synthesis by inhibiting bacterial DNA-gyrase and topoisomerase IV in Gram-negative and Gram-positive organisms respectively, and (B) azithromycin, which is a macrolide antibiotic that binds to 23S rRNA in the 50S subunit blocking translocation (1, 11).

A

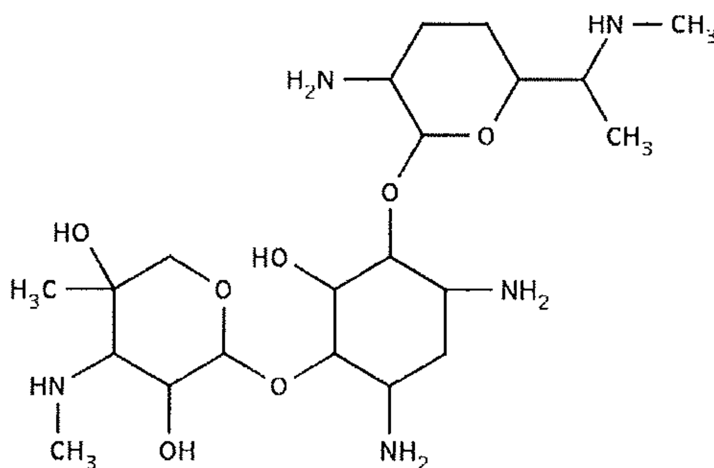


B

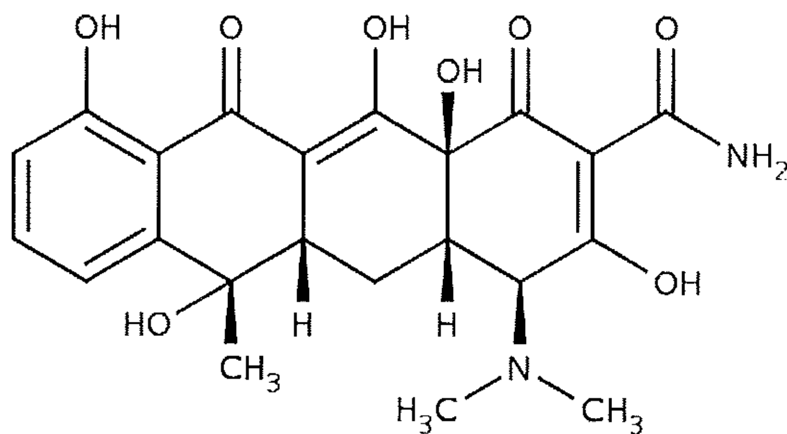


Appendix C: Molecular structures of (A) gentamicin, which is an aminoglycoside that directly inhibits protein synthesis by binding to the 30S ribosomal subunit, and causing the misreading of mRNA, and (B) tetracycline, which binds to the 30S ribosomal subunit, and inhibits the binding of aminoacyl-tRNA molecules to the ribosome (1, 11).

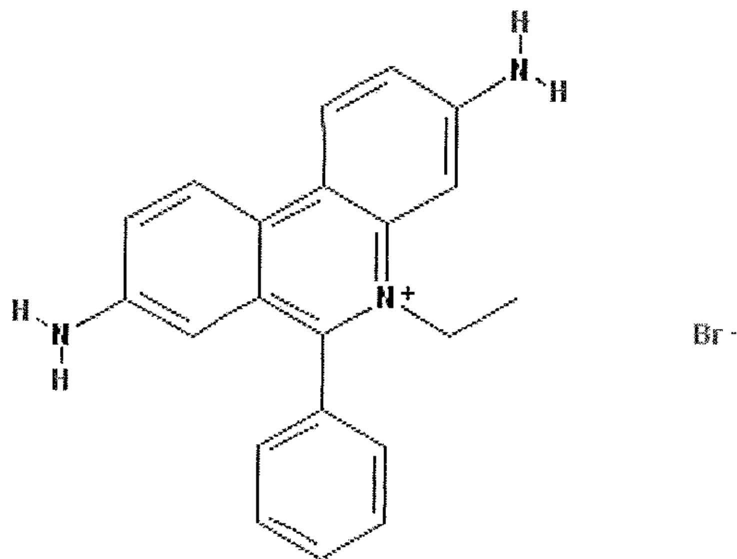
A



B



Appendix D: Molecular structure of ethidium bromide, which is a universal efflux pump substrate, and can also interfere with DNA replication (30).



Appendix E: Molecular structure of chlorhexidine, which is a disinfectant and topical anti-infective agent. It is often used in mouthwashes to prevent oral plaque (11).