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## Abstract

The spread of antibiotic resistant genes is of great public health concern. Wastewater treatment plants have been identified as a possible reservoir for these genes. This review gives an overview on the spread of antibiotic resistant genes in wastewater, based on existing data on occurrence of antibiotic as well as mechanisms involved in transfer and spread of resistant genes in wastewater systems. Dispersal of antibiotic resistant genes are facilitated by bacteriophages and mobile genetic elements, such as integrons, plasmids and transposons found in bacteria. Bacteriophage have been found to be extremely important in the spread of resistance genes in wastewater environment. This is due to their abundance in wastewater as well as their ability to transfer and acquire bacteria genes. Studies has shown that antibiotics are present in varying concentrations in distinct wastewater domains such as WWTPs. According to existing data, bacteria in wastewater exhibit resistance phenotype, using resistance mechanisms such as target by-pass, modification of target site, decreased permeability, efflux pumps amongst others. Studies has also shown that plasmids, bacteriophages, integrons and transposons involved in transfer of antibiotic resistant genes, as well as genes encoding other functions are prevalent in wastewater. In vitro studies performed on mobile genetic elements isolated from wastewater showed that mobile elements transmit resistance. The resistant genes are transferred via processes such as conjugation, transduction and transformation. To sum up, antibiotics are present in wastewater domains, and act as selective pressure for the development of resistance in some bacteria. Association of plasmids, integrons, transposons and bacteriophages with bacteria disperses resistant genes in wastewater. Due to the ability of genetic elements to acquire and transfer resistant genes among bacterial population.

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## Table of Contents

Abstract .....	ii
Acknowledgement.....	iii
Table of Contents .....	iv
List of Figures.....	vi
List of Abbreviations.....	viii
1. INTRODUCTION .....	1
2. Wastewater and its Classification .....	2
2.1 Domestic Wastewater .....	2
2.2 Industrial Wastewater .....	3
3. Antibiotics and Their Occurrence in WWTPS .....	4
3.1 Occurrence of Antibiotics in WWTPs .....	5
3.2 Occurrence of Frequently Detected Antibiotics in WWTPs .....	6
3.2.1 Quinolones (Fluoroquinolones).....	6
3.2.2 $\beta$ -lactams .....	7
3.2.3 Macrolides .....	9
3.2.4 Sulfonamides .....	10
3.2.5 Tetracyclines.....	11
4. Antibiotic Resistance and its Origin.....	12
4.1 Mechanism of Antibiotic Resistance .....	13
4.1.1 Modification of Antibiotic .....	16
4.1.2 Decreased Membrane Penetration and Efflux.....	17
4.1.3 Modification of Target Site.....	19
4.1.4 Target Bypass.....	20
5. Genetic Mechanism of Antibiotic Resistance .....	20
5.1 Mutation.....	20
5.2 Horizontal Gene Transfer .....	24
5.2.1 Conjugation .....	24
5.2.2 Transformation.....	27
5.2.3 Transduction.....	28
6. Mode of Spreading of Antibiotic Resistance in Wastewater.....	30
6.1 Antibiotic Resistant Genes (ARGs).....	30
6.2 Antibiotic Resistant Bacteria (ARBs).....	34
6.3 Bacteriophage .....	35

6.4 Integrons .....	38
6.5 Plasmid .....	42
6.6 Transposon .....	44
Conclusion .....	47
Future Perspectives .....	48
REFERENCES .....	49

## List of Figures

Figure 1 Structure of quinolone .....	6.
Figure 4: Cephalosporin Antibiotic Structure .....	<b>Error! Bookmark not defined.</b>
Figure 5: Structure of Erythromycin Antibiotic.....	10
Figure 6: Structure of Sulfamethoxazole.....	11
Figure 7: Tetracycline Structure.....	11
Figure 8: Bacterial Mechanism of Resistance to Different Class of Antibiotics.....	14
Figure 9: Schematic Illustration Showing Five Families of Efflux Pumps in Bacteria with Examples of Each.....	18
Figure 10: Simplified Representation of Conjugation Process.....	25
Figure 11: Transformation Mechanism.....	28
Figure 12: Generalized Transduction.....	30
Figure 13: Specialized Transduction.....	30
Figure 14: Illustration of The Lytic and Lysogenic Cycles in Phages.....	36

List of Tables

Table 1 Percentage of domestic water use.....	3 . 4
Table 2 Classification of some constituents found in wastewater .....	6
Table 3 Occurrence of some antibiotics in WWTPs.....	6
Table 4 Resistance Mechanism of Some Frequently Used Antibiotics.....	.15
Table 5 Antibiotic Resistance Genes Identified in MWTPs.....	33

## List of Abbreviations

ARB - Antibiotic Resistant Bacteria  
ARG - Antibiotic Resistant Gene  
DNA - Deoxy Ribonucleic Acid  
HGT - Horizontal Gene Transfer  
IS - Insertion Sequence  
MGE - Mobile Genetic Element  
MIC - Minimum Inhibitory Concentration  
MMR - Mismatch Repair  
MWTPs - Municipal Wastewater Treatment Plants  
PCR - Polymerase Chain Reaction  
PFU - Plaque- Forming Unit  
RNA- Ribonucleic Acid  
UWTPs - Urban Wastewater Treatment Plants  
WWTPs - Wastewater Treatment Plants



## 1. INTRODUCTION

Antibiotics are chemical substances produced by microorganisms, mainly soil bacteria that impede or kill other organisms (Cano & Colomé, 1988). The wide use of antibiotics in human medicine, veterinary and animal husbandry on a global scale, has led to the development of antibiotic resistance in certain bacteria (Gullberg et al., 2011; Kim, Jensen, Aga, & Weber, 2007) as well as the detection of antibiotics in water, sediment, animal and plant (Xu et al., 2015; T. Zhang, 2016; Zhou et al., 2013).

Antibiotic resistance is a natural occurrence which arises when infection caused by bacteria cannot be treated with appropriate dosage of an antibiotic (Friedman, Temkin, & Carmeli, 2016; Jose L. Martinez, 2014). According to World Health Organization (2017), the emergence of antibiotic resistant genes (ARGs) and antibiotic resistant bacteria (ARBs) is a global problem as it threatens public health. Bacteria acquire resistance phenotype by mutation, horizontal gene transfer (HGT) and by possessing inborn antibiotic resistance. Mutation involves the alteration of DNA sequence in a bacterial cell which results in genetic variation, whereas HGT has to do with movement of genetic information between living things (Burmeister, 2015). HGT is achieved via three main mechanism this includes transduction, transformation and conjugation (Munita & Arias, 2016). Mobile genetic elements (MGE) such as plasmids, integrons, and transposons play key roles in the transfer of ARGs in the environment (Kristiansson et al., 2011). In addition, recent studies have shown that bacteriophages also contribute significantly in horizontal spread of resistant genes (Balcazar, 2014; Muniesa, Imamovic, & Jofre, 2011; Rizzo et al., 2013).

Most of the antibiotics administered to both humans and animals are either partly or not metabolized, so that they end up as by-product in sewage (Hirsch, Ternes, Haberer, & Kratz, 1999). Wastewater treatment plants (WWTPs) receives wastewater from various sources such as housing units, hospitals, schools and industries (T. Zhang, 2016), resulting in WWTPs containing varying contaminants such as bacteria, metals, antibiotics, chemicals, antiseptics etc which can create a selective pressure favouring the development of ARGs and ARBs (Karkman, Do, Walsh, & Virta, 2018). Consequently, WWTPs has been identified as a prime source of pollutants such as antibiotics, heavy metals, ARGs and ARBs in to domains (T. Zhang, 2016), Unfortunately the traditional treatment process employed in WWTPs are

unable to completely remove antibiotics as well as resistant determinants present (Batt, Bruce, & Aga, 2006).

studies have been carried out on the occurrence of antibiotics, ARGs and ARBs in wastewater (Chen & Zhang, 2013; T. Zhang, 2016; T. Zhang & Li, 2011). Providing adequate knowledge on the issue but more information is needed on the fate and rate of spreading of these pollutants into the environment.

This review summarizes the spread of antibiotic resistant genes in wastewater from previous studies with emphasis on the mechanisms and modes of dissemination .

## 2. Wastewater and its Classification

The term wastewater is used for water originating from domestic or industrial activities that cannot be discharged in to the environment, due to the risk they pose to environment, public health etc (Madigan et al., 2006). Wastewater may consist of pollutants such as detergents, bacteria, nitrate, organic matter, chemical and metal with their organic fraction consisting mainly of carbon, hydrogen, oxygen and sometimes nitrogen (Tchobanoglous, Burton, Stensel, Metcalf, & Eddy, 2003). The concentration of the organic fraction of any wastewater determines the strength of that wastewater (Mara, 2004). Wastewater is classified into domestic and industrial wastewater based on its source. Wastewater is conveyed via sewers into WWTPS where they are treated using the appropriate treatment technique. The treated wastewater is called effluent (Madigan et al., 2006) and can be reused for certain activities such as agriculture, tourism etc. (Maryam & Büyükgüngör, 2017).

### 2.1 Domestic Wastewater

Domestic wastewater is wastewater generated from household, hospital and institutions consisting mainly of black water i.e. faecal water from toilets and grey water which includes water from bath tubs, washers and kitchen sinks (Mara, 2004). Domestic water use varies in the quantity of usage with flushing of toilets accounting for the highest water usage as shown in Table 1. Fresh domestic wastewater may be converted to septic wastewater when it loses its dissolved oxygen content (Davis & Masten, 2009), the fresh wastewater is grey in colour and has no odour whereas the septic wastewater has a black colour with an offensive odour (Davis & Masten, 2009).

Table 1. *Percentage of Domestic Water Use*

<b>Domestic Water use</b>	<b>Percentage use</b>
Flushing of toilets	40
Laundry	15
Bathing	30
Kitchen	10
Other use	5

*Note.* Sourced from Davis, M. L., & Masten, S. J. (2009). *Principles of environmental engineering and science* (2nd ed. ed.). Boston: McGraw-Hill.

Domestic wastewater is complex in nature due to the various types of pollutants it contains such as bacteria, viruses, pesticides, chemicals, detergents etc. Its content is unsafe as it contains a high level of disease causing organisms for example *Escherichia coli* (Mara, 2004). The quantity of organic matter in this type of wastewater is a key parameter for the effective treatment of domestic wastewater as it determines the degree of biological treatment employed in the treatment process (Maier, Pepper, & Gerba, 2009). Test such as biochemical oxygen demand (BOD) are carried out to determine the organic matter content of the wastewater (Mara, 2004).

Hospital as well as the consumption of antibiotics at home are one of the major sources of antibiotics in domestic wastewater (Dincer & Yigittekin, 2017). The combining of hospital wastewater with domestic wastewater without chemical or biological pre-treatment has resulted in the inadequate removal of medicine from wastewater after conventional treatment (Dincer & Yigittekin, 2017). Thus promoting the spread of resistance in the environment if discharged into surface and ground water (Dincer & Yigittekin, 2017).

## 2.2 Industrial Wastewater

Industrial wastewater as the name implies is generated from industrial activities. The type of pollutants present in industrial wastewater are diverse since different industries produce varying pollutants based on their operations (Davis & Masten, 2009). The constituents of the wastewater are classified as conventional, non-conventional and emerging pollutants (Tchobanoglous et al., 2003) as shown in Table2.

Industrial wastewater of high toxicity produced by individual industries undergo on-site pre-treatment before they are released in to the WWTPs (Tchobanoglous et al., 2003), this

is carried out to avoid damaging the sewer system conveying wastewater to the WWTPs and also from obstructing the treatment process in WWTPs (Tchobanoglous et al., 2003) .

Table 2. *Classification of Some Constituents Found in Wastewater*

<b>Conventional</b>	<b>Nonconventional</b>	<b>Emerging</b>
BOD	Metals	Antibiotics
COD	Surfactants	Steroid hormones
TSS	VOC	Home product
Bacteria	TDS	

*Note.* BOD= Biochemical oxygen demand, COD= Chemical oxygen demand, TSS= Total suspended solids, VOC= Volatile organic compound, TDS= Total dissolved solid, Sourced from Tchobanoglous, G., Burton, F. L., Stensel, H. D., Metcalf, & Eddy. (2003). *Wastewater engineering: treatment and reuse* (4th ed. revised by George Tchobanoglous, Franklin L. Burton, H. David Stensel. ed.). Boston: McGraw-Hill.

### 3. Antibiotics and Their Occurrence in WWTPS

Antibiotics are chemical substances used in the treatment and prevention of bacterial infection in both humans and animals (Gao et al., 2012; Kümmerer, 2009). Almost all the antibiotics available today are naturally derived from soil inhabiting bacteria such as the actinomycetes (Tortora, Funke, & Case, 2004), which uses the produced antibiotic to compete with other microorganism (Sosa et al., 2010).

Antibiotics can be classified in to numerous classes based on chemical structure this includes,  $\beta$ -lactams, glycopeptides, polymixins, macrolides, oxazolidinones, quinolones, sulphonamides, streptogramins, tetracyclines etc (Díaz-Cruz & Barceló, 2005) with antibiotics belonging to the same class having similar characteristics in terms of activity. The potency of the various classes of antibiotics depends mainly on their pharmacokinetic and pharmacodynamic properties (Yilmaz & Özcengiz, 2017).

Antibiotics are termed either as broad spectrum or narrow spectrum based on the range of bacteria they affect (Cano & Colomé, 1988). They are called broad spectrum when they affect a wide group of bacteria and narrow spectrum when they affect limited bacteria, for example only gram- positive bacteria (Tortora et al., 2004). Antibiotics can be bactericidal

in that they carry out their mode of action by killing bacteria, this includes antibiotics such as penicillin, fluoroquinolones etc. whereas bacteriostatic antibiotics such as tetracycline, macrolides, sulphonamides etc. work by preventing bacteria from multiplying.

Antibiotic has been seen to attack bacteria cell via five modes of action namely, by inhibition of cell wall synthesis, inhibition of protein synthesis, inhibitors of nucleic acid synthesis, inhibition of metabolic pathway and disruption of bacterial membrane structure (Tenover, 2006).

The utilization of antibiotic in present day medicine began with the discovery of artificial antibiotics obtained from dyes (Bosch & Rosich, 2008). The first natural antibiotic called penicillin was discovered in 1928 by Alexandra Fleming, and naturally formed antibiotics from plants were first utilized in medical science *during Paul Ehrlich`s study in 1908 (Dincer & Yigittekin, 2017)* all these discoveries contributed significantly to the transformation of the present day medical science.

### 3.1 Occurrence of Antibiotics in WWTPs

Antibiotics have been discharged in to WWTPS for decades from sources such as households, hospitals and industries i.e. pharmaceutical industry (T. Zhang, 2016), as a result many classes of antibiotics have been globally discovered in different WWTPs (T. Zhang, 2016). Nine classes of antibiotics such as macrolides, quinolones,  $\beta$ -lactams, sulphonamides, tetracycline lincosamides, reductase inhibitor (trimethoprim), glycopeptide and amphenicol have been detected in the influents and effluents of different WWTPs around the world (Tran, Reinhard, & Gin, 2018). The occurrence of fluoroquinolones, macrolides, sulphonamides, and trimethoprim in WWTPs was global (Tran et al., 2018), whereas tetracycline,  $\beta$ -lactams, glycopeptide and amphenicol were more prevalent in WWTPs of developing countries such as China, Thailand etc (Minh et al., 2009; Tran et al., 2018). This may be due to the absence of restriction on the purchase and extensive use of antibiotics in these countries compared to developed countries such as Sweden, Canada etc where restrictions are placed on the purchase and use of antibiotics in certain activities.

According to Zhang (2016) and Tran et al. (2018), the concentration of antibiotics in WWTPs depends on certain factors such as the consumption pattern of antibiotics, population

density and size, type of sewer system, weather variations and the efficiency of the treatment plant.

Table 3. Occurrence of some antibiotics in WWTPs

Antibiotic	Location	Influent Concentration (ng/l)	Effluent Concentration (ng/l)
Penicillin G	Australia	<2	<2
Sulfamethoxazole	USA	1090	210
Sulfamethoxazole	China	5450-7910	<100
Sulfamethoxazole	Korea	450	<30
Sulfamethoxazole	Mexico	390	0.31
Sulfamethoxazole	Sweden	<80-674	<80-304
N <sup>4</sup> -sulfamethoxazole	Switzerland	850-1600	<20-180
Trimethoprim	Mexico	0.59	180
Trimethoprim	USA	0.14-1.10	<50-550
Trimethoprim	UK	213-300	218-322
Trimethoprim	Sweden	80	40
Trimethoprim	China	120-320	120-230
Tetracycline	USA	200	<30
Tetracycline	China	96-1300	180-620
Ciprofloxacin	USA	<50-310	<50-60
Ciprofloxacin	Sweden	90-300	<6-60
Ciprofloxacin	Australia	90	130
Ciprofloxacin	China	80	27
Erythromycin	USA	<50-1200	<50-300
Erythromycin	Taiwan	226-1537	361-811
Erythromycin	China	470-810	520-850
Erythromycin	UK	71-141	145-290
Erythromycin	Switzerland	60-190	60-110

Note. Sourced from Le-Minh, N., et al., *Fate of antibiotics during municipal water recycling treatment processes*. Water Research, 2010. 44(15): p. 4295-4323.

### 3.2 Occurrence of Frequently Detected Antibiotics in WWTPs

#### 3.2.1 Quinolones (Fluoroquinolones)

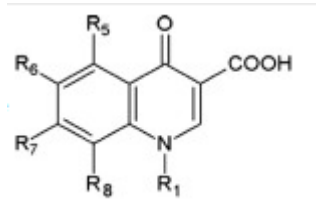


Figure 1: Structure of Quinolone, Reprinted from Van Doorslaer, X., et al., *Fluoroquinolone antibiotics: An emerging class of environmental micropollutants*. Science of The Total Environment, 2014. 500-501: p. 250-269.

Quinolones and fluoroquinolone belong to a class of antibiotics obtained from nalidixic acid, and are administered in the treatment of bacterial infections (Jia, Wan, Xiao, & Hu, 2011). The general structure of Quinolones is shown in figure 1. Nalidixic acid was the first quinolone used and it was seen to have antibacterial effect on some organism (Andersson & MacGowan, 2003). Fluoroquinolones are a class of quinolones that possess a fluorine ring, examples of such includes ciprofloxacin, norfloxacin etc (Figure 2).

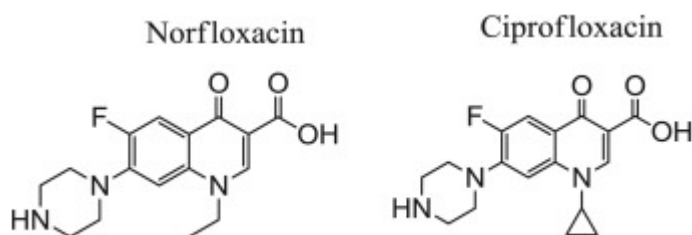


Figure 2. Structure of Norfloxacin and Ciprofloxacin Antibiotic, Reprinted from Tegze, A., et al., *Degradation of fluoroquinolone antibiotics during ionizing radiation treatment and assessment of antibacterial activity, toxicity, and biodegradability of the products*. Radiation Physics and Chemistry, 2018. 147: p. 101-105.

According to Kolpin et al. (2002) and Jia et al. (2011) the presence of quinolone has been detected in water bodies, and its presence in WWTPs corresponds with the global wide use of quinolones (T. Zhang, 2016). Quinolones such as pipemidic acid, nalidixic acid belonging to the first generation and moxifloxacin, gatifloxacin belonging to the fourth generation antibiotics have been identified in WWTPs (T. Zhang & Li, 2011). High concentrations of quinolones have been recorded in some countries for example, in Hong Kong and China 460 ng/l and 370 ng/l of norfloxacin was detected in the influent of WWTPs respectively (T. Zhang & Li, 2011). Significant amounts of quinolones are removed from WWTPs by adsorption to sludge as they are not easily biodegraded (Le-Minh, Khan, Drewes, & Stuetz, 2010; Lindberg et al., 2006).

### 3.2.2 $\beta$ -lactams

$\beta$ -lactams are the most widely used antibiotics in human medicine, they are also used in animal husbandry to promote growth (Cha, Yang, & Carlson, 2006). This class of antibiotics possess a beta-lactam ring in their structure and the mode of action is by inhibition of cell wall synthesis (Madigan et al., 2006). They consist of two sub classes namely penicillin and cephalosporin, with a ring system merged to a  $\beta$ -lactam ring as the main distinguishing feature (Cha et al., 2006) as seen in Figure 3 and 4.

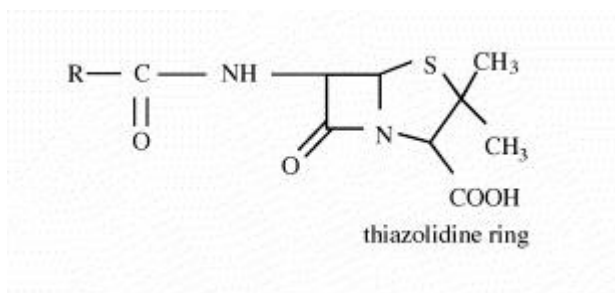


Figure 2. Structure of the Penicillin Antibiotic, Reprinted from Cha, J., S. Yang, and K. Carlson, *Trace determination of  $\beta$ -lactam antibiotics in surface water and urban wastewater using liquid chromatography combined with electrospray tandem mass spectrometry*. Journal of Chromatography A, 2006. 1115(1-2): p. 46-57.

Amoxicillin, cefaclor and cephalexin according to studies have been identified as the most predominant type of  $\beta$ -lactams in WWTPs (Watkinson, Murby, & Costanzo, 2007; T. Zhang & Li, 2011). Due to the global frequent use of  $\beta$ -lactams their concentrations have been seen to be more in influents and less or completely absent in effluents (Le-Minh et al., 2010). The low concentration of  $\beta$ -lactams in effluents is due to the ring system being unstable, so that it undergoes either chemical or enzyme hydrolysis during treatment (Kümmerer, 2009; Le-Minh et al., 2010; Watkinson et al., 2007). For example a WWTP in China recorded 153  $\mu\text{g/l}$  in the influent and 1.68  $\mu\text{g/l}$  in the effluent (Le-Minh et al., 2010; D. Li et al., 2008).

A significant quantity of  $\beta$ -lactams is removed by biodegradation in WWTPs (Zhang, 2016), this was the case of cephalexin which was seen to reduce from 2000 ng/l to 78 ng/l in a WWTPs in Australia (Costanzo, Murby, & Bates, 2005; Le-Minh et al., 2010).

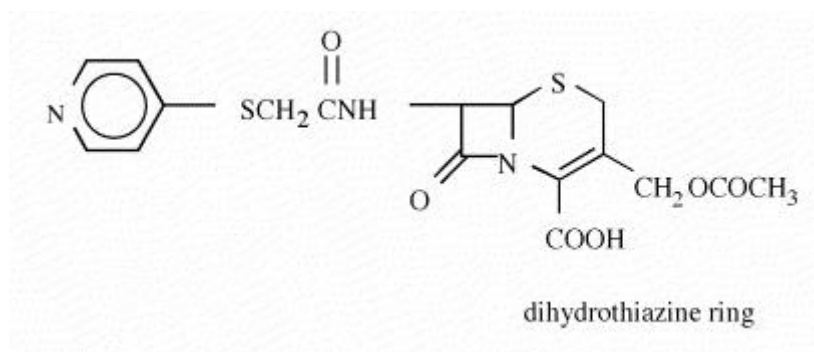


Figure 4. Cephalosporin Antibiotic Structure, Reprinted from Cha, J., S. Yang, and K. Carlson, *Trace determination of  $\beta$ -lactam antibiotics in surface water and urban wastewater using liquid chromatography combined with electrospray tandem mass spectrometry*. Journal of Chromatography A, 2006. 1115(1-2): p. 46-57.



### 3.2.3 Macrolides

Macrolide is one of the major classes of antibiotics used in modern medicine. They are made up of a large lactone ring as shown in Figure 5, which is interchanged with sugars and groups such as hydroxyl, alkyl and ketone to the nucleus (Göbel, Thomsen, McArdell, Joss, & Giger, 2005a; T. Zhang & Li, 2011). The mechanism of action for this class of antibiotics is the inhibition of protein synthesis in bacteria (Madigan et al., 2006). Macrolides are discharged from the body mainly in their original form and released into the sewer system (Hirsch et al., 1999; Le-Minh et al., 2010). The discharge of macrolides from the body in their original form increases the concentration of macrolide in the receiving WWTP, thus countries with a high consumption rate will have a high concentration of this antibiotic in the WWTP and vice versa (Le-Minh et al., 2010).

Erythromycin is one of the most used macrolides in hospitals (Kirst, 2002; Le-Minh et al., 2010), it is unstable under acidic conditions and converted to a metabolite called Erythromycin-H<sub>2</sub>O (Le-Minh et al., 2010). At a pH range of 6.5 to 8.0 both erythromycin and its metabolite can exist (Le-Minh et al., 2010). Based on studies Erythromycin-H<sub>2</sub>O is said to occur more than Erythromycin in WWTPs (T. Zhang & Li, 2011).

The metabolite along with six other macrolides have been detected in WWTPs globally (T. Zhang & Li, 2011) with 10025 ng/l of Erythromycin-H<sub>2</sub>O detected in influent (Kasprzyk-Hordern, Dinsdale, & Guwy, 2009; T. Zhang & Li, 2011) and 4330 ng/l in the effluent (Minh et al., 2009; T. Zhang & Li, 2011). Some of the macrolides detected in low frequency in WWTPs include, roxithromycin, clarithromycin, azithromycin, tylosin and oleandomycin (T. Zhang & Li, 2011).

Biodegradation and adsorption can be used for the removal of some quantities of macrolides from WWTPs, but these methods are still not efficient in their total removal (Göbel, McArdell, Joss, Siegrist, & Giger, 2007; Le-Minh et al., 2010).

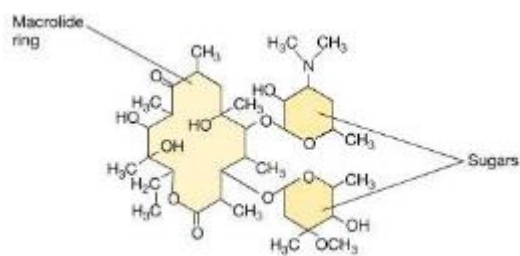


Figure 5. Structure of Erythromycin Antibiotic, Reprinted from Madigan, M.T., et al., *Brock Biology of Microorganisms, Global Edition: UEL*. 2006: Pearson Education M.U.A.

### 3.2.4 Sulfonamides

Sulfonamides are antibiotics that possess the sulfonamide functional group and they belong to the broad-spectrum antibiotics (Madigan et al., 2006). In a bacteria cell sulphonamide inhibits folic acid synthesis which eventually inhibits the synthesis of nucleic acid (Le-Minh et al., 2010). Sulfonamides are not entirely metabolized when ingested and are excreted either in the original form or as a metabolite into the sewer system (Göbel et al., 2005a; Le-Minh et al., 2010). The most excreted metabolites include, N4acetylsulfamethoxazole and other N4-acetylated sulphonamides which according to Göbel et al. (2005a) and Le-Minh et al. (2010) can be converted back to their parent compounds during treatment in WWTPs.

Sulfamethoxazole has been identified as the most frequently detected sulphonamide in WWTPs globally (Brown, Kulis, Thomson, Chapman, & Mawhinney, 2006; Le-Minh et al., 2010; T. Zhang & Li, 2011), with a concentration of 5597 ng/l in influent and 6000 ng/l in effluent (Batt et al., 2006; Peng, Tan, Tang, Yu, & Wang, 2008; T. Zhang & Li, 2011). Other sulphonamides detected frequently in WWTPs includes sulfadiazine, sulfapyridine, sulfamethazine etc (T. Zhang & Li, 2011).

The removal of sulfonamides from WWTPs is achieved to a certain level by adsorption and biodegradation, with adsorption accounting for most of the removed sulphonamides (Le-Minh et al., 2010).

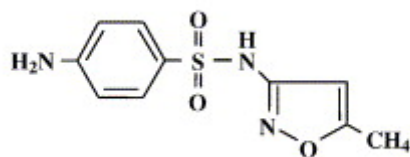


Figure 6. Structure of Sulfamethoxazole, Reprinted from Peng, X., et al., *A preliminary study on the occurrence and behaviour of sulfonamides, ofloxacin and chloramphenicol antimicrobials in wastewaters of two sewage treatment plants in Guangzhou, China*. *Science of The Total Environment*, 2006. **371**(1): p. 314-322.

### 3.2.5 Tetracyclines

Tetracyclines are bacteriostatic antibiotics that inhibit both gram-positive and gram-negative bacteria and are the second widely used antibiotics in human medicine (Madigan et al., 2006). They possess the naphthacene ring system and inhibit protein synthesis in a bacteria cell by interfering with the 30S ribosome function (Madigan et al., 2006).

The extensive use of tetracycline in the treatment of bacterial infections in humans, veterinary medicine as well as in animal production in some countries (Madigan et al., 2006), has resulted in the frequent detection of tetracycline in wastewater (Kim et al., 2007; Le-Minh et al., 2010). For instance, in the USA tetracycline was detected in concentration ranging between 0.1 and 0.6 µg/l in the influent of a WWTP (Kim, Eichhorn, Jensen, Weber, & Aga, 2005; Kim et al., 2007; Le-Minh et al., 2010).

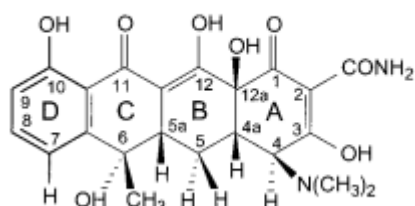


Figure 7. Tetracycline Structure, Reprinted from Halling-Sørensen, B., G. Sengeløv, and J. Tjørnelund, *Toxicity of Tetracyclines and Tetracycline Degradation Products to Environmentally Relevant Bacteria, Including Selected Tetracycline-Resistant Bacteria*. *Archives of Environmental Contamination and Toxicology*, 2002. **42**(3): p. 263-271

#### 4. Antibiotic Resistance and its Origin

According to WHO (2015) bacteria is said to be antibiotic resistance when therapeutic levels of antibiotic used to treat the infection it produces becomes ineffective. This is due to the bacteria undergoing certain changes which could either be natural or acquired. Antibiotic resistance is a natural process that has been in existence for a long time (Friedman et al., 2016), it can be traced as far back to the origin of antibiotics (Friedman et al., 2016; Shlaes et al., 1997) as most antibiotics are produced naturally by environmental bacteria such as soil bacteria (Jose L. Martinez, 2014). Antibiotic producing microorganisms developed a resistant mechanism to prevent the produced antibiotic from harming them (P. Courvalin, 2016), this mechanism of resistance was not a cause of concern back then as it had little or no effect on both humans and animals. As time progressed the use of antibiotics increased leading to a growing concern of antibiotic resistance. This concern was remedied by the development of new classes of antibiotics to counter the resistance mechanism (Jose L. Martinez, 2014). Presently due to the extensive and random use of antibiotics in medicine and animal husbandry (Kim et al., 2007; Rodríguez-Rojas, Rodríguez-Beltrán, Couce, & Blázquez, 2013). Antibiotic resistance has become a global threat to public health (World Health Organization, 2014), as an increasing number of pathogenic bacteria possesses the resistance mechanisms thus making the treatment of their infections difficult.

Microorganisms can become resistant to antibiotic either naturally or by acquisition of the resistant trait or gene (Munita & Arias, 2016). Natural antibiotic resistance can be illustrated using antibiotic producing microorganism such as soil bacteria. This type of bacteria need to protect themselves from the harmful substances they produce, and as such naturally develop resistance mechanism to enable them with stand the effect of the antibiotic they produce (Cox & Wright, 2013; Munita & Arias, 2016).

Gram negative bacteria possess outer membrane that shields them from harmful substances (P. Courvalin, 2016; Cox & Wright, 2013). For example, the outer membrane of gram-negative bacteria prevents the flow of  $\beta$ -lactam molecules to the intracellular region upon exposure (Madigan et al., 2006). Thus, allowing the bacteria to survive the harmful attack and become naturally resistant to  $\beta$ -lactam. There are a few environmental bacteria such as mycoplasma species that lack cell wall, these types of bacteria are more likely to survive an attack by

antibiotic such as penicillin which works by impeding cell wall (Madigan et al., 2006). Consequently, making this class of bacteria naturally resistant to the antibiotic applied.

Microorganism are said to have acquired antibiotic resistance when they undergo a genetic change that results in the mutant gene carrying antibiotic resistance (Madigan et al., 2006). The resistance gene is either picked up from the environment, or by transfer from one bacterium to another of the same specie or distinct species (Madigan et al., 2006). Antibiotic resistance is spread through the environment by three main agents this includes genes, bacteria and mobile genetic elements such as plasmids and transposons (P. Courvalin, 2016). The main source of antibiotic resistance genes has been attributed to environmental bacteria which are the key producers of antibiotics (Jose L. Martinez, 2014).

Mutation and HGT are the main processes that has led to the emergence of antibiotic resistance (Madigan et al., 2006; Munita & Arias, 2016; T. Zhang, 2016). Mutation is the alteration of genetic material which can result to different types of antibiotic resistance such as resistance that modifies antibiotic target site (MacGowan & Macnaughton, 2017; Jose L. Martinez, 2014). HGT involves the transfer of resistance genes originating mainly from environmental bacteria (P. Courvalin, 2016; Jose L. Martinez, 2014). In HGT genes are transferred from one bacterium called the donor to another known as the recipient (Madigan et al., 2006). This process is triggered of by very low concentration of antibiotic in the bacteria cell environment (Jose L. Martinez, 2014). Mobile genetic elements such as plasmids, transposons and integrons are essential for horizontal transfer of resistance genes from one organism to another (Munita & Arias, 2016).

#### 4.1 Mechanism of Antibiotic Resistance

Over the years with the wide use of antibiotics, bacteria especially pathogenic ones have developed mechanisms to enable them with stand antibiotic attacks (MacGowan & Macnaughton, 2017). From a biochemical approach, bacteria have adopted several mechanisms in surviving antibiotic attack. This includes modification of the antibiotic, decreased membrane penetration and efflux, modification of target site and target by pass (Holmes et al., 2016; Munita & Arias, 2016). Bacteria can use any one of the mechanism in Figure 8 to survive antibiotic attack, while some bacteria are specialized and are able to employ two or more mechanisms in surviving a specific antibiotic attack (Deutscher & Friedman, 2010; Munita & Arias, 2016).

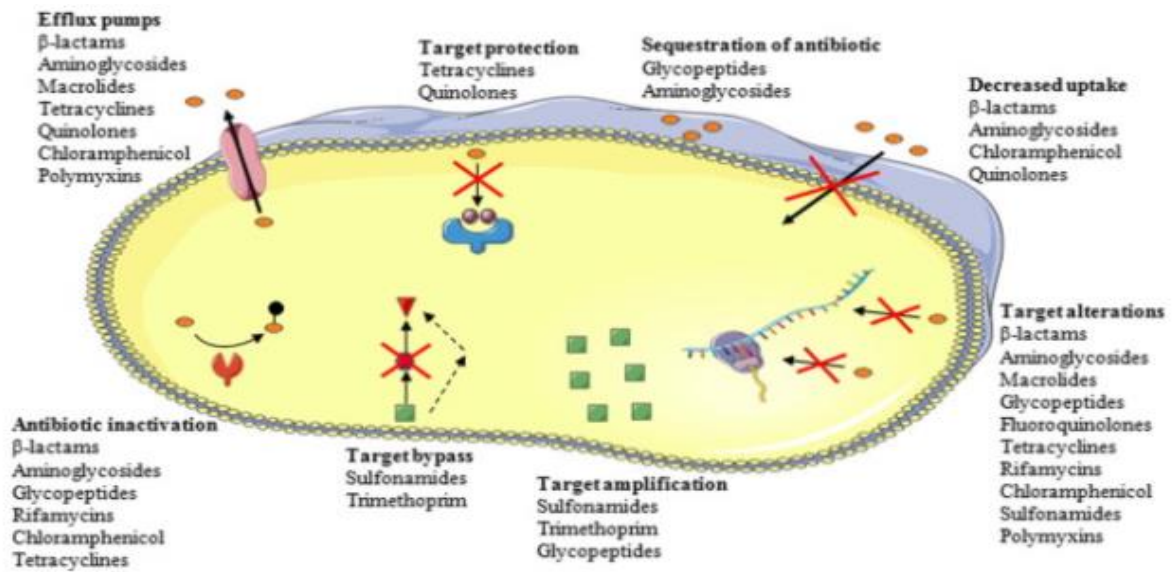


Figure 8. Bacterial Mechanism of Resistance to Different Classes of Antibiotics, Reprinted from Yılmaz, Ç., & Özcengiz, G. (2017). Antibiotics: Pharmacokinetics, toxicity, resistance, and multidrug efflux pumps. *Biochemical Pharmacology*, 133, 43-62. doi:<https://doi.org/10.1016/j.bcp.2016.10.005>

Table 4. Resistance Mechanism of Some Frequently Used Antibiotics

Antibiotic Class	Type of Resistance	Resistance Mechanism	Example
Macrolides	Altered target	Methylation of ribosome active site	erm-encoded methylases in <i>staphylococcus aureus</i>
	Efflux pumps	Mef type pump	<i>Streptococcus pneumoniae</i>
β-lactams	Altered penicillin binding protein	PBP2a	Mec A in <i>staphylococcus aureus</i>
	Enzymatic degradation	Penicillinase	Gram-negative bacteria
Quinolones	Altered target site	Mutation resulting to reduced binding to target site	Mutations in gyr A in gram-negative bacteria and in <i>staphylococcus aureus</i>
	Efflux	Membrane transporters	Mutations in gyr A and par C in <i>Streptococcus pneumoniae</i>
Tetracycline	Efflux	Membrane transporters	tet genes encoding efflux proteins in gram-positive and gram-negative bacteria
	Altered target	Production of proteins that bind to the ribosome and alter the target site	tet (M) and tet (O) in gram-positive and gram-negative bacteria
Aminoglycoside	Decreased uptake	Changes in outer membrane	<i>Pseudomonas aeruginosa</i>
	Enzymatic modification	Aminoglycoside modifying enzymes	Gram-negative bacteria

Note. Sourced from Kapoor, G., Saigal, S., & Elongavan, A. (2017). Action and resistance mechanisms of antibiotics: A guide for clinicians. *Journal of Anaesthesiology, Clinical Pharmacology*, 33(3), 300-305. doi:10.4103/joacp.JOACP\_349\_15

#### 4.1.1 Modification of Antibiotic

Resistance due to the modification of antibiotics involves the production of an enzyme by the bacteria under attack (Deutscher & Friedman, 2010). The produced enzyme can modify the antibiotics either by chemical alteration or by the destruction of the antibiotic molecule (Deutscher & Friedman, 2010; Munita & Arias, 2016). In the case of chemical alteration, resistance is attained when the enzyme catalyses reactions such as acetylation, phosphorylation and adenylation (Dzidic, Suskovic, & Kos, 2008; Munita & Arias, 2016), which decreases the binding of the antibiotic molecule to the target site. The antibiotic type typically affected by this mechanism are antibiotics that function by inhibition of protein synthesis (Munita & Arias, 2016). For instance, in aminoglycoside antibiotic resistance the enzyme transferases catalyses either acetylation, phosphorylation or adenylation reaction, resulting in the altered aminoglycoside antibiotic affected in the way it binds to target site (Dzidic et al., 2008). Enzymatic modification has accounted for most of the global aminoglycoside antibiotic resistance recorded with the enzymes housed mainly by plasmids and transposons (Munita & Arias, 2016).

Some enzymes are capable of catalysing more than one reactions and these enzymes are said to be bifunctional (Munita & Arias, 2016). Most gram-positive bacteria exhibit the bifunctional ability in attaining resistance, an example is the *Enterococcus sp* which has developed resistance to gentamicin antibiotic using its bifunctional ability (Munita & Arias, 2016).

$\beta$ -lactam is a typical example of modification of antibiotic by enzymatic destruction of the antibiotic as seen in Table 4. Here the  $\beta$ -lactam antibiotic losses its potency as  $\beta$ -lactamases destroys the amide bond of the  $\beta$ -lactam ring present in the structure of  $\beta$ -lactam antibiotics. For instance, *Staphylococcus aureus* produces  $\beta$ -lactamases enzyme which hydrolysis the  $\beta$ -lactam ring present in  $\beta$ -lactam antibiotics (Deutscher & Friedman, 2010; MacGowan & Macnaughton, 2017; Munita & Arias, 2016), resulting in the resistance of *Staphylococcus aureus* to  $\beta$ -lactam.



#### 4.1.2 Decreased Membrane Penetration and Efflux

In this mechanism there is a change in permeability of porins which transports materials to the cell region mainly by diffusion (Munita & Arias, 2016), this change in porin occurs without altering the porin structure (Jose L. Martinez, 2014). Changes in porins such as damage to the porin structure, porin size and copy number of porin decreases permeability of porins, (Dzidic et al., 2008; Kapoor, Saigal, & Elongavan, 2017; Kumar & Schweizer, 2005), thus reducing intracellular penetration of antibiotics such as quinolones, b-lactams and other hydrophilic compounds (Munita & Arias, 2016). Porins are classified in to several groups based on their function this includes, general porins, outer membrane porins and specific porins (Fernández & Hancock, 2012). General porins are the most important with respect to extrusion of antibiotic, as they are responsible for the specification of porin size to hydrophilic substances (Fernández & Hancock, 2012).

Since most antibiotics exert their effect within a cell, bacteria able to reduce the amount of antibiotic entering the cell may develop resistance. This resistance is usually of low level concentration to such antibiotic (Deutscher & Friedman, 2010; Munita & Arias, 2016). For example, the presence of outer membrane in gram-negative bacteria has led to decreased quinolone antibiotic penetration in some gram-negative bacteria, due to a change in membrane permeability (Munita & Arias, 2016). Most of the acquired resistance in *Pseudomonas aeruginosa* to antibiotics is a result of decreased membrane permeability (Kapoor et al., 2017).

According to MA Webber and Piddock (2003), Dzidic et al. (2008) and Jose L. Martinez (2014) another mechanism of resistance is the extrusion of antibiotics from within a cell to the environment, by protein membranes called efflux pump. Efflux pumps are present in most bacteria and can affect virtually all classes of antibiotics (Dzidic et al., 2008; Munita & Arias, 2016; MA Webber & Piddock, 2003). Antibiotics mostly affected by this mechanism are those exerting intracellular effects for example tetracycline, fluoroquinolone etc (Dzidic et al., 2008). The genes coding for efflux pump proteins can be found in chromosomes or plasmids of bacterial cell (Sun, Deng, & Yan, 2014).

Efflux pumps are grouped in to five families which include the major facilitator super family (MFS), multidrug and toxic compound extrusion (MATE) family, the resistance-nodulation-cell division (RND) family, the small multidrug resistance (SMR) family and the adenosine

triphosphate (ATP)- binding cassette (ABC) super family (Dzidic et al., 2008; Munita & Arias, 2016; MA Webber & Piddock, 2003). The various groups of efflux pumps vary in their mechanism of operation, structure, energy source and type of antibiotic they act on (Dzidic et al., 2008; Munita & Arias, 2016; Sun et al., 2014). Efflux pumps are also categorized in to single- component system or multi-component system owing to the number of transporter each efflux pump possesses (Sun et al., 2014). Most efflux pumps found in gram-positive bacteria belong to the single component system whereas most efflux pumps in gram-negative bacteria belong to the multi-component system (Fernández & Hancock, 2012). Pumps can act either on a specific antibiotic (drug specific) or on a wide range of antibiotic (multiple drug specific), with multiple drug efflux pump enhancing multiple drug resistance in certain bacteria (Dzidic et al., 2008; MA Webber & Piddock, 2003). The expression of the distinct types of efflux pumps are controlled by local and global regulators which are normally located adjacent to the genes encoding efflux protein (Sun et al., 2014).

The tetracycline efflux pump is one of the first efflux system studied and provides a good insight on the efflux mechanism (Munita & Arias, 2016). In this case, tetracycline is transported out of the cytoplasmic membrane of a bacteria such as *Escherichia coli*, via the tetracycline efflux pump in to the environment. Consequently, resulting in *E. coli* surviving exposure to tetracycline (Munita & Arias, 2016).

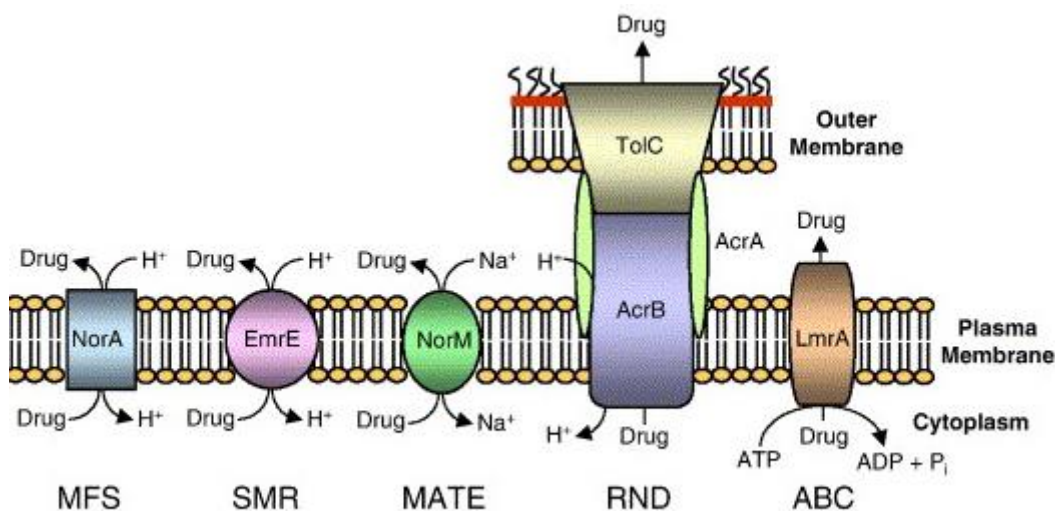


Figure 9. Schematic Illustration Showing the Five Families of Efflux Pumps in Bacteria with Example of Each, Reprinted from Kumar, A., & Schweizer, H. P. (2005). Bacterial resistance to antibiotics: Active efflux and reduced uptake. *Advanced Drug Delivery Reviews*, 57(10), 1486-1513. doi:<https://doi.org/10.1016/j.addr.2005.04.004>

#### 4.1.3 Modification of Target Site

Modification of target site involves tactics such as protection of the antibiotic target site and alteration of the antibiotic target site by bacteria (Munita & Arias, 2016) so that they become resistance to antibiotic. In the protection of target site, a resistance protein produced by the bacteria cell either displaces the antibiotic molecule, alters binding site, or may bind to the antibiotic target site (W. Li et al., 2013; Munita & Arias, 2016). Therefore, preventing the antibiotic from exerting its effect so that resistance to such antibiotic is exhibited.

In the tetracycline resistance by *Campylobacter jejuni*, tetracycline resistance protein Tet (O) binds to target ribosome so that tetracycline molecule is unable to bind (W. Li et al., 2013; Munita & Arias, 2016). The displacement of the tetracycline molecule by Tet (O) protein prevents tetracycline from exerting its effect, so that protein synthesis is not affected and *Campylobacter jejuni* is able to survive tetracycline attack (Munita & Arias, 2016). Another example of this mechanism is the quinolone resistance protein QnrA found in some bacteria cell (Munita & Arias, 2016). This protein protects the bacterial DNA from the harmful effect of quinolone antibiotic which targets the DNA gyrase and topoisomerase iv enzyme (Jose L. Martinez, 2014).

Modification of target site in a bacteria cell can arise from alteration such as RNA polymerase alteration (Lahiri et al., 2016). An example is the rifampicin antibiotic resistance in *Mycobacterium tuberculosis*, which occurs due to mutation of the RNA polymerase (Lahiri et al., 2016). The mutation occurs at the  $\beta$  subunit of the RNA polymerase which is the rifampicin target site in a bacteria cell and this target site is encoded by the rpoB genes (Lahiri et al., 2016). Resistance arising from the above mutation enables *Mycobacterium tuberculosis* to survive rifampicin attack with the altered target site still able to perform its function of transcription (Munita & Arias, 2016). A high level of rifampicin resistance has been recorded in *Mycobacterium tuberculosis* due to single step point mutation which substitutes some amino acid in the rpoB genes (Munita & Arias, 2016).

Alteration in penicillin- binding protein (PBP) accounts for most of the antibiotic resistance in gram-positive bacteria (Kapoor et al., 2017) with resistance arising from reduced affinity of the antibiotic molecule (Dzidic et al., 2008). An example is the methicillin resistance in *Staphylococcus aureus*, which arises due to acquisition of a new gene (mecA) in the

chromosome of *Staphylococcus aureus* (Kapoor et al., 2017; Munita & Arias, 2016). The new *mecA* gene acquired produces a new penicillin-binding protein called PBP2a which is highly resistant to most  $\beta$ -lactam antibiotic effect. Consequently, reducing affinity with  $\beta$ -lactam and as such continues to perform its function (Dzidic et al., 2008; Kapoor et al., 2017).

#### 4.1.4 Target Bypass

Bypass of target site is another tactic which involves the production of many target sites by bacteria to overcome antibiotic effect (Munita & Arias, 2016). An example is the resistance to trimethoprim-sulfamethoxazole antibiotics in *E. coli*, which occurs due to the over production of DHPS and DHRF target enzymes by mutation in genes (Eliopoulos & Huovinen, 2001; Munita & Arias, 2016). The presence of many target molecules overcomes the trimethoprim-sulfamethoxazole molecule rendering it ineffective at inhibiting folate synthesis (Munita & Arias, 2016).

### 5. Genetic Mechanism of Antibiotic Resistance

Mutation and horizontal gene transfer (HGT) are two major genetic processes that have led to the emergence of antibiotic resistance. Mutation of genes encoding target site, access and protection pathways can confer resistance phenotype to a bacterium (Ishizawa, Ying, Tsuru, & Yomo, 2015; J. L. Martinez & Baquero, 2000). Although the rate at which mutation occurs in bacteria is low, the rapid generation time exhibited by bacteria enhances the spread of antibiotic resistance. Horizontal gene transfer (HGT) is the movement of genes among microorganisms belonging to either same or distinct species (Huang et al., 2017). This mechanism is carried out by bacteria through three major processes such as conjugation, transduction and transformation (Cano & Colomé, 1988).

#### 5.1 Mutation

Mutation is the alteration of heritable genetic information (DNA) of an organism (Cano & Colomé, 1988; Madigan et al., 2006). This process can arise due to error in DNA replication (Deutscher & Friedman, 2010; Ishizawa et al., 2015) as well as from exposure to mutagens. DNA replication error can ensue from failure in base selection, proof reading and mismatch repair (MMR) system which affects accuracy of DNA replication (J. L. Martinez & Baquero, 2000; Woodford & Ellington, 2007). Mutation can also occur due to oxidative and alkylation processes (Woodford & Ellington, 2007) and from wrongly repaired DNA of a bacteria cell (Dzidic et al., 2008). The process of mutation takes place mostly in actively dividing cell

(Dzidic et al., 2008; J. L. Martinez & Baquero, 2000) whereas certain mutation such as adaptive mutation can occur in non-dividing cells (J. L. Martinez & Baquero, 2000).

The bacteria emerging from mutation is called a mutant and is usually affected by the process either positively or negatively. Mutations that affect the cell positively give the mutant a selective advantage enabling mutant bacteria to thrive in adverse environment (Woodford & Ellington, 2007). Also accumulation of mutation in a bacterial population brings about variety which enhances their adaptation to environment (Woodford & Ellington, 2007). The occurrence of some mutation affect mutant bacteria adversely by diminishing fitness, although this can be rectified by secondary site mutations (Schulz zur Wiesch, Engelstädter, & Bonhoeffer, 2010).

Antibiotic resistance emerging from mutation is due to mutation in genes encoding antibiotic target, access and protection pathways in a bacterial cell (J. L. Martinez & Baquero, 2000), which increases minimum inhibitory concentration (MIC) of the antibiotic (Ishizawa et al., 2015). When a bacterial population is exposed to a certain antibiotic, a part of that population is susceptible to that antibiotic. However, some bacteria are able to undergo mutation which enables them survive exposure to the given antibiotic (Munita & Arias, 2016). The resulting mutant is said to have a resistance phenotype so that it has a selective advantage and can reproduce to increase the population. Consequently, resulting in new population that exhibits resistance to the specific antibiotic (Munita & Arias, 2016).

The MMR system in a bacteria cell is responsible for identifying DNA mispairing and activation of DNA repair cascade to fix errors (Woodford & Ellington, 2007), since faults in repair systems of bacteria makes such cell prone to mutation (Ishizawa et al., 2015; Woodford & Ellington, 2007). Furthermore, the absence of MMR system increases the occurrence of mutation as well as recombination frequency of that bacterium (Rodríguez-Rojas et al., 2013). Such a bacterium is said to be hypermutable and exhibits a mutator phenotype, which varies in strength from one bacterium to another (Dzidic et al., 2008; Woodford & Ellington, 2007). Mutator phenotype genes such as mut S, mut H and mut U have been identified in some microorganism such as *E. coli* and *S. enterica*. Mutators gradually gather useful mutation (Ishizawa et al., 2015) and show increased mutation rate of approximately

10,000 times (J. L. Martinez & Baquero, 2000). This mutation rate varies for individual mutators as it is a function of their distinct mutator alleles (Ishizawa et al., 2015). Mutators are also capable of possessing varieties of alleles which enables them to defy antibiotic attack (J. L. Martinez & Baquero, 2000).

Since mutation is a genetic change that leads to antibiotic resistance, most organisms have developed resistance to certain antibiotics by mutation of the genes encoding resistance (Deutscher & Friedman, 2010; Jose L. Martinez, 2014). Several genes in a bacteria cell may be required for bacteria to develop resistance to an antibiotic owing to the presence of many antibiotic target sites or protection pathways in such bacteria (Dzidic et al., 2008).

Various forms of mutation have been seen to convey different levels of resistance in several organisms for instance; *Mycobacterium tuberculosis* is resistant to most antibiotic therapy especially mono antibiotic therapy is due to chromosomal mutations (Deutscher & Friedman, 2010; Dzidic et al., 2008; Woodford & Ellington, 2007). This form of mutation confers resistance to *M. tuberculosis* either by altering target sites or by excess production of target sites in the cell (Dzidic et al., 2008). Bacteria that acquire resistance by chromosomal mutation tend to have a regular resistance pattern which makes it possible to identify appropriate drugs for effective treatment of their infections, therefore this form of mutation is of great importance in medical science (Dzidic et al., 2008). A point mutation in the nucleotide of a bacteria cell is another form of mutation that leads to antibiotic resistance in bacteria such as *E. coli* by modifying the function of the gene encoding resistance (Chattopadhyay & Sokurenko, 2013; Dzidic et al., 2008).

Adaptive mutation results to resistance in bacteria due to alteration in genes which are triggered by stressors present in the bacteria environment (Fernández & Hancock, 2012). This type of mutation can occur in a slowly or non-dividing cell unlike most mutations that occur only in actively dividing cells (Dzidic et al., 2008). Bacteria thrive in their host or ecological niche by adjusting to changes in the environment and still being able to perform their basic functions (Munita & Arias, 2016). Stressors such as exposure to a non-lethal dose of antibiotic, pH change etc. can trigger mutation in a bacteria cell resulting in resistance (Fernández & Hancock, 2012), such resistance is temporal since it can be reversed when the stressor causing it is removed from the bacteria environment (Fernández & Hancock, 2012).

According to J. L. Martinez and Baquero (2000), frequency of mutation in a bacterial population is the measure of all mutant bacteria present. The frequency of mutation leading to antibiotic resistance differ from one bacterium to the other, since different mechanism are employed by bacteria in obtaining resistance to antibiotic (Normark & Normark, 2002). An example of bacteria species exhibiting the same resistance to  $\beta$ -lactam antibiotic at different frequency is seen in *Enterobacter cloacae*, which produces  $\beta$ -lactamase enzyme at a high frequency. Nevertheless, in *E. coli* chromosomal mutation producing  $\beta$ -lactamase enzyme occurs at a low frequency (Normark & Normark, 2002). Also, frequency of mutation leading to the inactivation of a resistance gene in a bacteria cell is higher than mutation which conveys resistance by alteration of gene (Normark & Normark, 2002).

Several factors affect the mutation rate of bacteria either by increasing or decreasing it. Mutability is the likelihood of mutation to produce a resistance phenotype and this affects mutation rate in terms of number of genes and gene structure (J. L. Martinez & Baquero, 2000). Gene structure affects mutability in that the number of sub units prone to mutation in genes encoding resistance varies from gene to gene. Therefore, mutation leading to antibiotic resistance corresponds to the number of those subunits available in the gene structure (J. L. Martinez & Baquero, 2000). Consequently, genes containing more mutation prone subunits have a higher mutation rate and vice versa (J. L. Martinez & Baquero, 2000). For example, *E. coli* quinolone resistance occurs due to changes in about seven subunits of *gyrA* gene, whereas in the *parC* gene change occurs in only three subunits. As a result, *gyrA* has a higher mutation rate than *parC* (J. L. Martinez & Baquero, 2000; M. Webber & Piddock, 2001).

Number of genes encoding for resistance in the bacterial cell influences mutability value since mutation in one or different genes can result to resistance phenotype in a bacterium (J. L. Martinez & Baquero, 2000). Therefore, mutability for resistance owing to mutation in only one gene in a bacterium is low whereas a high mutability is obtained for resistance phenotype due to mutation in several gene (J. L. Martinez & Baquero, 2000). Thus, affecting mutation rate as increase in mutability increases mutation rate and vice versa.

Location of genes in the chromosome of some bacteria belonging to the *Enterobacteriaceae* family is seen to affect mutation rate, as genes located far from replication origin has a mutation rate about two times that of genes near the origin of replication (J. L. Martinez &

Baquero, 2000). Other factors affecting mutation rate includes bacteria stress, antibiotic concentration in selective window, presence of transposable element in a bacteria and large variety of resistance genes (J. L. Martinez & Baquero, 2000). Reversal of mutator allele and acquisition of suppressor mutation affect mutation rate adversely by reducing it (Dzidic et al., 2008).

## 5.2 Horizontal Gene Transfer

Horizontal gene transfer is the movement of genetic information between organisms of different species (Huang et al., 2017). This process is important in the spread of antibiotic resistance determinants between bacteria (Barlow, 2009) and plays a huge role in the evolution of prokaryotes. Due to most prokaryotes acquiring genes through this process (Boto, 2010). HGT occurs in some eukaryotes especially single celled eukaryotes (Boto, 2010; Huang et al., 2017) but recent studies by Huang et al. (2017) and Pace, Gilbert, Clark, and Feschotte (2008) shows that HGT can as well occur in multicellular eukaryotes such as humans. Three major mechanisms of HGT are transformation, transduction, and conjugation (Madigan et al., 2006).

### 5.2.1 Conjugation

Conjugation is one of the process of horizontal gene transfer involving the transfer of DNA from one bacterium cell to another, in which both cells are in contact (Arber, 2014; Madigan et al., 2006) see Figure 10. The bacterium giving away DNA is called the donor while the bacterium receiving the DNA is the recipient (Arber, 2014). This transfer is usually plasmid mediated and such plasmids are known as conjugative plasmids (Madigan et al., 2006). The conjugative plasmids are capable of transferring copies of themselves as well as copies of other plasmids in to new cells (Bennett, 2008), with the transfer methods differing from one plasmid to another (Madigan et al., 2006).



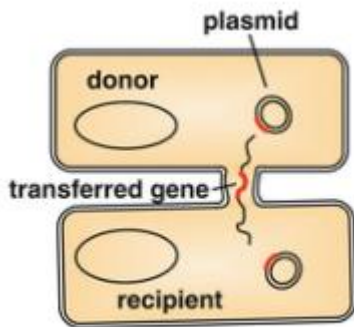


Figure 10. Simplified Representation of Conjugation Process, Reprinted from Blokesch, M. (2015). Protocols for Visualizing Horizontal Gene Transfer in Gram-Negative Bacteria Through Natural Competence. In *Hydrocarbon and Lipid Microbiology Protocols* (pp. 189-204): Springer.

Conjugative plasmid in gram- positive bacteria are smaller than those found in gram-negative bacteria. This difference in size makes their contact mechanism differ (Bennett, 2008) as well as different conjugative system in both gram-positive and gram-negative bacteria (Heuer & Smalla, 2007). Some plasmids are not self-transmissible but can be mobilized and transferred to a new bacterium cell, in the process of conjugation by the conjugative apparatus (Madigan et al., 2006; Thomas & Nielsen, 2005).

Two main steps achieve conjugation firstly, the formation of mating pair which links the donor and recipient cell together and secondly the transfer of single strand plasmid DNA to the recipient cell (Andrup, 1998). Fertility factor (F- plasmid) present in donor cells (Arber, 2014), consist of about forty genes present in the transfer region (Zatyka & Thomas, 1998). These genes function mostly in the formation of mating pair (Madigan et al., 2006). Some of the genes in the transfer region are used by the donor to produce external appendage called sex pili, which establishes contact with the receptor of the recipient cell (Bennett, 2008; Madigan et al., 2006). This brings the donor and recipient cell in contact, so that a pore which allows the movement of plasmid DNA as well as some encoded proteins of the donor to the recipient cell is created (Thomas & Nielsen, 2005). The transfer region may vary slightly from one plasmid to another consequently resulting to difference in pili structure (Madigan et al., 2006). Gram-negative conjugative plasmids have two types of pili which varies in function and size (Andrup, 1998). Long and flexible pili found in F- plasmid, IncD

plasmid and IncJ plasmid function efficiently in liquid medium whereas short and fixed pili present in IncN and IncP plasmid effectively transfer on solid surfaces (Andrup, 1998).

The second step process of conjugation commences as Tra I enzyme (Madigan et al., 2006) nicks of a single strand plasmid DNA from donor, and transfers to a recipient cell where a harmonious DNA strand is formed (Madigan et al., 2006; Zatyka & Thomas, 1998). As DNA strand is transferred to recipient cell, rolling circle replication occurs in donor cell replacing the transferred DNA. Thus at the end of the conjugation process both the donor and recipient have complete plasmid (Cano & Colomé, 1988; Madigan et al., 2006).

In transfer involving F- plasmids, the donor cell (F<sup>+</sup>) transfers to a recipient cell (F<sup>-</sup>), which lacks the F- plasmid. At the end of the transfer process both the donor and recipient cell contains the F- plasmid (Cano & Colomé, 1988; Madigan et al., 2006). Recipient cells that acquire F- plasmid become potential donors, since some bacteria cell can lose their F- plasmid during cell division (Cano & Colomé, 1988).

The process of conjugation is also used to transfer a part of the chromosome genes (Madigan et al., 2006). This is achieved by transposable elements such as insertion sequence (IS), which are present in both the F-plasmid and chromosome of certain bacteria such as *E. coli* (Madigan et al., 2006). Homologous recombination between similar insertion sequence in the F-plasmid and chromosome results to integration of F-plasmid in to the chromosome (Madigan et al., 2006). Consequently, leading to the transfer of a part of the chromosome genes (Madigan et al., 2006).

According to del Campo et al. (2012), frequency of conjugation is the proportion of the number of transconjugants to the number of either the donor or recipient. This frequency increase greatly by remixing mating populations (del Campo et al., 2012). In addition, antibiotic can affect conjugation frequency for example, the conjugational transfer of pUCP24T plasmid from *E. coli* to *Pseudomonas aeruginosa*. This transfer showed significant increase in conjugation frequency, as *E. coli* was treated with sub- minimal inhibitory concentration of either ciprofloxacin or levofloxacin (Shun-Mei et al., 2018).

The mechanism of conjugation is also utilized by pathogenic bacteria in the spreading of resistant genes (Patrice Courvalin, 1994). For instance, tetracycline resistant gene carried in the F- plasmid of *Neisseria gonorrhoeae* can be transferred to another bacterium cell via

conjugation (Cano & Colomé, 1988). Therefore, conjugation is an important mode of horizontal gene transfer which enhances the spread of antibiotic resistance among pathogenic bacteria in nature (del Campo et al., 2012).

In conclusion, the process of conjugation is used by bacteria in both acquiring and exchanging genes and requires contact between the two cells involved.

### 5.2.2 Transformation

Transformation is the uptake of free DNA by bacteria and the integration of the obtained DNA into their chromosomes (Cano & Colomé, 1988) see Figure 1. This process is an important mechanism by which bacteria obtain genetic information from different species (Thomas & Nielsen, 2005). Bacteria capable of performing this process are said to be naturally competent (Heuer & Smalla, 2007; Thomas & Nielsen, 2005). Thus, natural competence is a physiological state which allows efficient uptake of DNA and is genetically managed (Heuer & Smalla, 2007). Over eighty species of both gram-positive and gram-negative bacteria are naturally competent (Blokesch, 2016), this includes several human pathogenic bacteria such as *Campylobacter*, *Streptococcus* and *Pseudomonas* (Thomas & Nielsen, 2005). Non-competent or poorly transformable bacteria such as *E. coli* can be artificially induced into competency, by treatment with calcium chloride solution or by undergoing the process of electroporation (Blokesch, 2016; Madigan et al., 2006).

Competency is attained in most bacteria in their exponential growth phase (Cano & Colomé, 1988). Nevertheless, not all bacteria of the same species are able to display competency (Blokesch, 2016). Several factors such as starvation, DNA damage, antibiotic stress and intracellular growth triggers the onset of competency in bacteria (Blokesch, 2016). For example, competency in *Streptococcus pneumoniae* is instigated by damaged DNA and the presence of antibiotics (Blokesch, 2016). Quorum sensing is an occurrence where by cells in a bacteria population, send signals to each other by releasing pheromone when high cell density is reached (Synder & Champness, 2003). This phenomenon is used by some bacteria such as *Vibrio cholerae* to attain competency (Blokesch, 2016).

Free DNA necessary for transformation can be made available in the environment by bacteria releasing DNA into the environment, lysing of bacteria as well as from the decomposition of dead organisms (Thomas & Nielsen, 2005). DNA from decaying organisms

becomes exposed and available for uptake after the process of cell lysis (Thomas & Nielsen, 2005).

In the process of transformation, free DNA is taken up by a bacterium and bound to the outer cell layer. Translocation to the cytoplasm occurs and then obtained DNA is integrated into the chromosome by homologous recombination (Heuer & Smalla, 2007; Madigan et al., 2006; Synder & Champness, 2003). Several genes are involved in the process of transformation and perform different functions, this includes production of DNA uptake machinery, protection of the newly acquired DNA amongst others (Blokesch, 2016). Most bacteria can take up DNA of any type and integrate it into their chromosomes. Nevertheless, some are discrete in DNA uptake such as *Haemophilus influenzae* owing to their fixed DNA sequence (Synder & Champness, 2003).

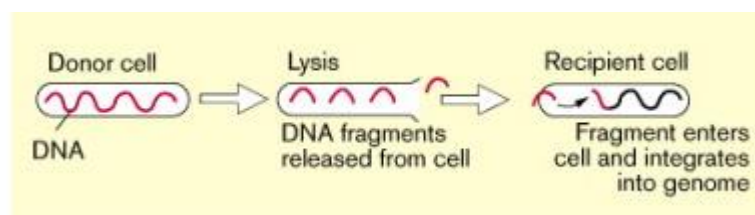


Figure 11. Transformation Mechanism, Reprinted from Saunders, N. J., Hood, D. W., & Moxon, E. R. (1999). Bacterial evolution: Bacteria play pass the gene. *Current Biology*, 9(5), R180-R183. doi:[https://doi.org/10.1016/S0960-9822\(99\)80108-0](https://doi.org/10.1016/S0960-9822(99)80108-0)

The persistence of free DNA in the environment for a relatively long time, ascertains bacteria transformation frequency as well as bacterial exposure time (Thomas & Nielsen, 2005). This Frequency varies among bacteria of the same species and consequently different efficiencies in the integration of DNA that is taken into chromosomes (Heuer & Smalla, 2007).

To sum up, the process of transformation is essential in bacteria existence as it enables bacteria to acquire and exchange genes. Thus, contributing to their evolution, enhances adaptation as well as brings about diversity to the bacterial population.

### 5.2.3 Transduction

Transduction is another horizontal gene transfer mechanism in which bacterial DNA from a host infected bacterium cell, is transferred to another bacterium by bacteriophage (Heuer & Smalla, 2007). Bacteriophages are profuse in nature and can infect a wide range of bacteria

in the environment (Lupo, Coyne, & Berendonk, 2012). The presence of DNA packaging mechanism in a bacteriophage, enables the phage to package DNA belonging to its host bacterium during reproduction (Madigan et al., 2006; Synder & Champness, 2003). Phages carrying such features are said to be transducing (Cano & Colomé, 1988; Synder & Champness, 2003) as they can carry out the process of transduction. Host DNA can mistakenly be packaged in to viral DNA by the bacteriophage packaging enzyme during viral reproduction, with this error in packaging occurring mostly during lytic infection (Madigan et al., 2006).

Transducing bacteriophages can be virulent or temperate based on their reproductive cycle (Madigan et al., 2006). They are classified as either specialized or generalized phages (Cano & Colome,'1988). Generalized transducing phages reproduce inside their host bacteria evolving to viral progeny (Cano & Colome,'1988) . This breaks the cytoplasm of the host bacteria so that as the phages continue to reproduce, fragments of the host bacteria can be mistakenly packaged in to some new phages (Cano & Colome,' 1988). Nevertheless, specialized phages integrate in to their host chromosome to reproduce and may pick up a portion of their host DNA as they exit the chromosome (Cano & Colome,' 1988). Therefore, generalized transduction involves the transfer of genes from any area of the host chromosome to the recipient bacteria (Madigan et al., 2006; Cano & Colome,' 1988). However, in specialized transduction only a few bacteria genes close to the incorporation site of the prophage can be transferred to another bacterium (Synder & Champness, 2003; Cano & Colome,' 1988) . Furthermore, frequency of specific transduction is more efficient and higher than that of generalized transduction (Madigan et al., 2006).

The mechanism of transduction has several benefits such as the absence of cell coupling, persistence of transducing phages in the environment and the protection of transported DNA by phages (Heuer & Smalla, 2007). Also, laboratory test has showed the process of transduction plays a vital role in the exchange of genetic materials among microorganism (Lupo, Coyne & Berendonk, 2012).

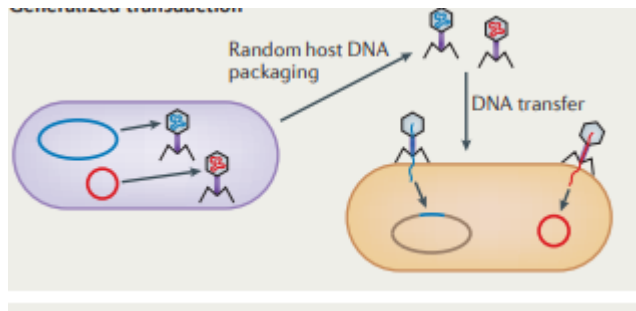


Figure 12. Generalized transduction, Reprinted from Salmond, G. P., & Fineran, P. C. (2015). A century of the phage: past, present and future. *Nature Reviews Microbiology*, 13(12), 777.

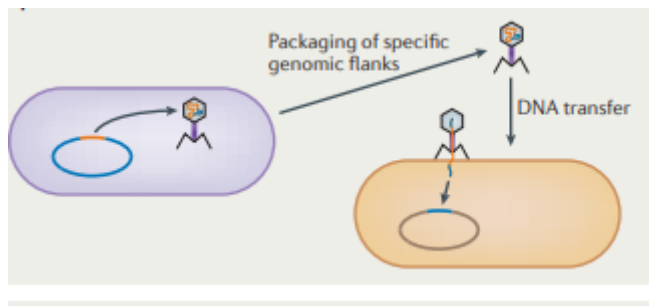


Figure 13. Specialized Transduction, Reprinted from Salmond, G. P., & Fineran, P. C. (2015). A century of the phage: past, present and future. *Nature Reviews Microbiology*, 13(12), 777.

## 6. Mode of Spreading of Antibiotic Resistance in Wastewater

### 6.1 Antibiotic Resistant Genes (ARGs)

The development of antibiotic resistant genes in present day environment can be linked to the wide use of antibiotic (Bouki et al., 2013). This has adversely affected both human and animal, as treatment of infections are made difficult owing to the development of antibiotic resistance in bacteria (Manaia et al., 2018; Bouki et al., 2013). This phenomenon of antibiotic resistance in bacteria is controlled genetically by ARGs (X.-X. Zhang, Zhang, & Fang, 2009). ARGs are usually produced by molecular mechanisms such as efflux pump, target bypass, modification of target site etc (X.-X. Zhang et al., 2009).

ARGs has been identified in most WWTPs based on several studies (Chen & Zhang, 2013; Manaia, 2014; Novo, André, Viana, Nunes, & Manaia, 2013; Rizzo et al., 2013), with community, hospital and relevant industry being the major sources of ARGs in to wastewater

treatment plants (Karkman et al., 2018). Municipal wastewater treatment plants (MWWTPs) usually receives wastewater from distinct sources such as hospitals, industry, homes etc (Karkman et al., 2018; Manaia, 2014), with the different wastewater containing varying pollutants such as antibiotic, heavy metals, bacteria from different environment (Karkman et al., 2018). During the treatment process in WWTPs antibiotic residues present are in continuous contact with bacteria, this leads to the development of resistant gene in some bacteria present (Bouki et al., 2013; X.-X Zhang et al., 2009). The presence of high bacteria cell density owing to nutrient availability and the selective pressure exerted by antibiotic residues or metals (Rizzo et al., 2013; Manaia, 2014; X.-X Zhang et al., 2009), creates a conducive state which enhances horizontal transfer of ARGs from one bacterium to another (Bouki et al., 2013; X.-X Zhang et al., 2009). Thus, making municipal wastewater treatment plants potential hot spot for the transfer of ARGs (Rizzo et al., 2013).

Polymerase chain reaction (PCR) and metagenomics are the frequently used techniques employed in analysing ARGs in wastewater (Karkman et al., 2018). Metagenomics has an advantage over PCR in that, it does not require previous knowledge on the sequence of the ARG to be studied whereas such knowledge is needed for PCR (Karkman et al., 2018). These methods have been used to identify ARGs present in wastewater. Although the actual transfer process of ARGs in wastewater is still not ascertained, due to lack of appropriate technique (Rizzo et al., 2013). The study of the spread of resistance genes in wastewater with PCR method is demanding. This is due to the presence of complex pollutants as well as humic substances, detergents etc. that can interfere with detection of certain bacterial species and genes (Rizzo et al., 2013).

Resistant genes such as tetracycline resistant genes, methicillin resistant genes, sulphonamide resistant genes etc have been identified in many WWTPs. Tetracycline (tet) resistant genes is seen to be the most occurring of these genes in wastewater with high rates in most countries (X.-X Zhang et al., 2009). See Table 5 for more ARGs found in WWTPs.

Selective pressure has been seen to be a key factor in the spreading of ARGs in aquatic environment, because an increase in this pressure enhances the acquisition of genes by bacteria (X.-X Zhang et al., 2009). Furthermore, the acquisition of genes by bacteria may improve fitness, such that spreading of genes becomes fast( X.-X Zhang et al., 2009). Several ARGs occur on mobile genetic elements such as plasmids, integrons and transposons (J. Li,

Cheng, Xu, Strong, & Chen, 2015). These genes located on mobile genetic elements are said to be more prone to spreading since they can easily be acquired horizontally by another bacterium when mobilized (Karkman et al., 2018). Also, some mobile genetic elements present in WWTPs can facilitate recombination and transfer of new antibiotic resistant genes at high cell density (X.-X Zhang et al., 2009).



Table 5. *Antibiotic Resistant Genes Identified in MWTPs*

Class of Antibiotic	Type of Mechanism	Example of gene
B- lactams	Target protection	mecA
	Drug modification	ampC
	Drug modification	IMP
Macrolides	Target protection	ermB
	Drug efflux	mel
Quinolones	Target protection	qnrA3
	Target protection	qnrS
	Target protection	qnrB1
Glycopeptides	Target modification	vanA
Aminoglycosides	Drug modification	aphA
	Drug modification	aadB
Tetracyclines	Drug efflux	tetA, tetB, tetD
	Target protection	tetM, tetS
	Drug modification	tetX
Sulfonamides	Target modification	sul1, sul2, sul3
Trimethoprim	Drug modification	dfrA1, dfrA12

*Note.* Sourced from Rizzo, L., Manaia, C., Merlin, C., Schwartz, T., Dagot, C., Ploy, M. C., . . . Fatta-Kassinos, D. (2013). Urban wastewater treatment plants as hotspots for antibiotic resistant bacteria and genes spread into the environment: A review. *Science of The Total Environment*, 447, 345-360. doi:<https://doi.org/10.1016/j.scitotenv.2013.01.032>

## 6.2 Antibiotic Resistant Bacteria (ARBs)

Bacteria from different environment are present in WWTPs due to the distinct sources of wastewater in WWTPs (Karkman et al., 2018; X.-X Zhang et al., 2009). The presence of antibiotic residues and metals in WWTPs exerts selective pressure, such that some bacteria can acquire resistant genes horizontally. Therefore, leading to the development of antibiotic resistant bacteria (Novo et al., 2013; Bouki et al., 2013). Thus, ARBs have been identified in both raw and treated wastewater samples on a large scale (Bouki et al., 2013), using either culture or molecular based methods.

Culture based methods used to analyse antibiotic resistance in WWTPs involves isolation of the bacteria to be studied on a media, and assessing the bacteria growth in response to certain antibiotic concentrations (McLain, Cytryn, Durso, & Young, 2016). However, molecular based method utilizes DNA or other molecular parts of a bacterial cell (Luby, Ibekwe, Zilles, & Pruden, 2016). This method is used for bacteria that play a part in antibiotic resistance but cannot be cultured or that multiply very slowly (Rizzo et al., 2013). Bacteria belonging to the phyla of proteobacteria, Actinobacteria, Firmicutes and Bacteroidetes are usually found in wastewater, and are not easy to be cultivated in labs (Novo et al., 2013). Thus, molecular based methods can be used in the study of antibiotic resistance in these bacteria.

Coliforms and enterococci bacteria are commonly used as indicators for faecal contamination (Turolla, Cattaneo, Marazzi, Mezzanotte, & Antonelli, 2018). Consequently, studies on antibiotic resistance in wastewater bacteria commonly utilizes these indicators (Manaia, 2014), with results of such studies normally indicating history of antibiotic resistant acquisition in the indicators (Manaia, 2014). For instance, studies by Rizzo et al. (2013) and Łuczkiwicz, Jankowska, Fudala-Książek, and Olańczuk-Neyman (2010) confirmed a high rate of antibiotic resistance within the range of, 20-40% in enterococci for tetracycline, erythromycin and quinolone antibiotics. In addition, to a low resistance rate of 1- 7% for sulphonamides and aminopenicillins. Also, in *E. coli* which is a coliform bacteria high antibiotic resistance rate in the range of 10- 40% was obtained for aminopenicillins, sulphonamides and tetracyclines, with a low rate for quinolones (Łuczkiwicz et al., 2010; Rizzo et al., 2013).

Antibiotic resistant bacteria of medical importance such as staphylococci methicillin-resistant bacteria (Volkman, Schwartz, Bischoff, Kirchen, & Obst, 2004) and *Pseudomonas aeruginosa* possessing multi-resistant (Rizzo et al., 2013), are usually found in hospital wastewater (Volkman et al., 2004; Rizzo et al., 2013). Consequently, hospital is said to be a major source of medically relevant ARBs into WWTPs (Rizzo et al., 2013).

Conventional treatment in WWTPs are unable to completely remove ARBs (Novo et al., 2013; Manaia, 2014; Rizzo et al., 2013), as concentration of some ARBs are seen to either increase remarkably, decrease or remain unchanged in the effluent (Manaia, 2014).

Moreover, treatment process in most WWTPs are incapable of reducing the rate of antibiotic resistance, since increase in antibiotic resistance of enterococci as well as in *E. coli* have been observed in effluent of most WWTPs globally (Rizzo et al., 2013).

### 6.3 Bacteriophage

Bacteriophage are viruses that infect bacteria (Muniesa, Colomer-Lluch, & Jofre, 2013). They are the most abundant life form on the biosphere, as they are found in virtually all environment in significantly large numbers (Muniesa et al., 2013). Bacteriophage normally consist of nucleic acid molecule, genome and a protein coat called capsid (Muniesa et al., 2013). They may also possess external structures such as tails and spikes which enhances persistence of bacteriophage in the environment (Muniesa et al., 2013).

Two types of bacteriophage exist that is virulent and temperate bacteriophage. This distinction is made solely to their different life cycle (Muniesa et al., 2013) see Fig 14. Virulent phages infect their host cell, multiply inside and release new phage particles (Balcazar, 2014), this reproduction pattern is termed lytic cycle and is particular to virulent phages (Muniesa et al., 2013). Temperate bacteriophage reproduces following the lysogenic cycle. In this reproduction pattern, the genome of the infecting phage stays in the host bacteria cell and replicates either independently or alongside with host cell after integration into the chromosome of the host bacteria (Muniesa et al., 2013).

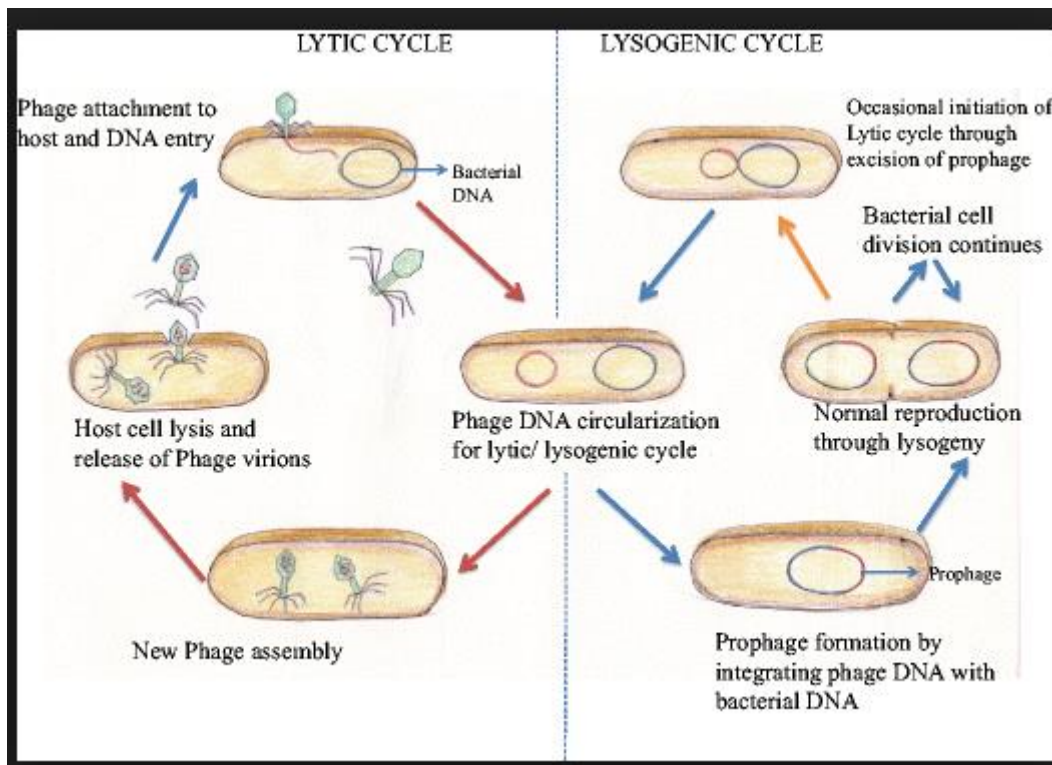


Figure 14. Illustration of the lytic and lysogenic cycle in phages, Reprinted from Sharma, S., Chatterjee, S., Datta, S., Prasad, R., Dubey, D., Prasad, R. K., & Vairale, M. G. (2017). Bacteriophages and its applications: an overview. *Folia microbiologica*, 62(1), 17-55.

Some bacteriophage can be host specific or infect only a narrow range of susceptible bacteria whereas some phages exhibit a wide host range (Muniesa et al., 2013). Phages of the *Phikmvlikevirus* genus and *Twortlikevirus* genus infect only bacteria belonging to the *Pseudomonas* genus and *staphylococcus* genus respectively (Subirats, Sánchez-Melsió, Borrego, Balcázar, & Simonet, 2016). Bacteriophages exhibiting broad host range includes phages of the *lambdalikeyirus* genus, which can interact with proteobacteria as well as firmicutes (Subirats et al., 2016).

Phages integrated into host cell and replicating are termed prophage (Muniesa et al., 2013). A prophage can be naturally or artificially induced to reproduce following the lytic cycle. The presence of ultraviolet light, specific antibiotics such as quinolone are capable of artificially inducing a prophage to reproduce with the lytic cycle (Muniesa et al., 2013). Nevertheless, in situations where host bacteria goes in to starvation existing prophage can be naturally induced to reproduce following the lytic cycle (Muniesa et al., 2013).

The ability of phages to mistakenly package host bacterial DNA during replication makes it possible for phages to carry out transduction (Synder & Champness, 2003). Bacterial genes

such as *psb* encoding for photosynthesis, *pho* encoding for phosphate acquisition, *stx* encoding shiga toxin etc are found in phages (Muniesa et al., 2013). Although these genes are not utilized by the phages, they can be transferred via transduction to distinct host bacteria (Muniesa et al., 2013). Consequently, conveying new functions to host cell (Lekunberri, Subirats, Borrego, & Balcázar, 2017).

Bacteriophage are abundant in wastewater and account for a bacteriophage fraction of about  $10^5$  to  $10^8$  plaque forming units of raw wastewater (Lood, Ertürk, & Mattiasson, 2017). The high concentration of bacteriophage, favourable gene transfer conditions, presence of resistant bacteria as well as resistant genes in wastewater environment enhances transduction of resistance genes in WWTPs (Lood et al., 2017).

Studies has revealed that bacteriophage acts as a pool of resistant genes (Lood et al., 2017; Muniesa et al., 2013). For example, the study done by Colomer-Lluch, Jofre, and Muniesa (2011) on samples from a WWTP and the receiving river revealed significant concentration of bacteriophage in both environment. Resistant genes such as *bla<sub>TEM</sub>* and *bla<sub>CTX-M</sub>* was identified in the phage DNA from both environment using the PCR method. Further analysis to determine if the resistant genes identified would convey resistance to host bacteria was abortive. This may have been due to unconducive environment as well as inability to find an ideal host (Colomer-Lluch et al., 2011). Nevertheless, the identified resistant genes were transduced in to a recipient *E. coli* which showed resistance to ampicillin (Colomer-Lluch et al., 2011).

Also, another study by Balcazar (2014) on the analysis of phage DNA obtained from distinct hospital wastewater as well as from effluent of a municipal WWTP was performed. Resistant genes such as *bla<sub>TEM</sub>*, *bla<sub>CTX-M</sub>*, *bla<sub>SHV</sub>*, *qnrA*, *qnrS* and *qnrB* were identified. In addition, 16SrDNA genes similar to those found in *Acinetobacter* and *Arcobacter* were identified in DNA of phages obtained from wastewater (Muniesa et al., 2011).

Phages such as Stx bacteriophage, Somatic coli phages and phages infecting bacteroides are amongst the phages usually found in raw wastewater (Muniesa et al., 2011). In the work carried out by Subirats et al. (2016) phages belonging to the order *Caudovirales* were detected in hospital wastewater. The phage DNA fraction contained high proportions of ARGs than those found in the bacterial DNA fraction. The ARGs encoded genes for both RND

and ABC efflux pump, resistance to  $\beta$ -lactam as well as resistance to quinolones (Subirats et al., 2016).

The persistence of phage DNA in the environment even under extreme conditions, owing to the protein capsid surrounding its DNA has been studied. Calero-Cáceres and Muniesa (2016) discovered that ARGs such as  $bla_{TEM}$ ,  $bla_{CTX-M}$  and  $sul1$  were present, in both phage DNA and bacteria DNA from the raw municipal wastewater. The ARGs were subjected to different temperature and Ph. ARGs present in the phage DNA was seen to persist more under the harsh conditions (Calero-Cáceres & Muniesa, 2016).

High transduction rate has been observed in-situ for certain phages normally found in wastewater, this includes *E. coli* phage. The study performed by Kenzaka, Tani, Sakotani, Yamaguchi, and Nasu (2007) observed high transduction rate for phages P1, T4 and *E. coli* phage. This phages exhibited high transduction rate when transducing  $bla$  gene to recipient *E. coli* bacteria. Transduction frequency of *E. coli* phage was within  $< 4 \times 10^{-9}$  to  $4 \times 10^{-8}$  per PFU, whereas that of phage P1 and T4 was in the range of  $3 \times 10^{-8} - 2 \times 10^{-6}$  per PFU and  $1 \times 10^{-8} - 4 \times 10^{-8}$  per PFU respectively (Kenzaka et al., 2007).

All in all bacteriophage can be said to play essential role in the spread of resistant genes among bacteria in wastewater environment. The ability of phages to carry bacterial genes and the persistence of these genes in the environment, makes transduction a viable process in the transfer of genes between bacteria of different species and biomes. Lastly, the presence of antibiotics, ultraviolet rays etc in WWTPs capable of inducing phages. In addition to the presence of phages with high in-situ transduction frequency in wastewater, could imply that transduction occurs in wastewater at a relatively high rate.

#### 6.4 Integrons

Integrons are genetic elements in bacteria capable of capturing and expressing genes lodged in gene cassettes (Stalder, Barraud, Casellas, Dagot, & Ploy, 2012). Integrons are found mostly in gram-negative bacteria (Stalder et al., 2012) such as gamma- proteobacteria (Moura, Pereira, Henriques, & Correia, 2012). However, gene cassettes are well distributed in gram-negative alpha- proteobacteria and are also found in Firmicutes and Archaea (Moura et al., 2012). Gene cassette can exist in both linear and circular shape (Stalder et al., 2012).

They are linear when integrated in integrons and circular when existing in their free non-replicating state (Stalder et al., 2012).

Integrons consist of three vital elements this includes promoter, *intI* gene encoding the enzyme integrase and an integration site *attI* (Moura et al., 2012; Stalder et al., 2012) all of which are essential for their integration with gene cassettes. Gene cassette able to integrate with an integron usually lacks a promoter but carries an *attC* site which can identify the integrase enzyme (Madigan et al., 2006). Integrons can obtain and express genes residing in gene cassette mainly due to their ability to integrate with gene cassette through the process of recombination (Madigan et al., 2006). Recombination process occurs between the *attC* site of the gene cassette and the *attI* site of the integron, resulting to the insertion of the gene cassette at the *attI* site (Stalder et al., 2012). The genes present in the gene cassette are then expressed by a promoter in the integron (Madigan et al., 2006; Moura et al., 2012).

Integrons are of two types that is chromosomal integron and mobile integron (Stalder et al., 2012). Chromosomal integron are found on chromosomes of bacteria mostly from the terrestrial or marine environment such as *Vibrio cholerae* (Stalder et al., 2012) and usually carry many gene cassettes ranging in hundreds (Madigan et al., 2006; Stalder et al., 2012). As a result, they are termed super integrons with the function of most of their genes being unknown (Madigan et al., 2006; Stalder et al., 2012).

Mobile integrons are widely distributed in gram-negative bacteria and are seldomly found in gram-positive bacteria (Stalder et al., 2012). Mobile integrons carry few gene cassettes which normally encodes resistance to antibiotics (Madigan et al., 2006; Stalder et al., 2012), so that they are sometimes referred to as resistant integrons (Stalder et al., 2012). Gene cassettes encoding resistance found in mobile integrons includes *catB* conferring resistance to chloramphenicol, *aacA4* encoding resistance to gentamicin and tobramycin, *arr3* conferring resistance to rifampicin, *bla<sub>OXA-2</sub>* and *bla<sub>GES-7</sub>*, encoding resistance to  $\beta$ -lactams and *aadA* conferring resistance to streptomycin and spectinomycin etc (Moura et al., 2012). Furthermore, *aadA* is said to be the most prevalent of these resistant gene cassettes in isolated bacteria (Moura et al., 2012).

Mobile integrons are classified in to five classes based on the amino acid sequence of the integrase enzyme. This includes class 1, class 2, class 3, class 4 and class 5 mobile integrons

(Stalder et al., 2012). Class 1 mobile integrons are more abundant in the environment followed by the class 2 and class 3 integrons whereas class 4 and class 5 integrons are rarely detected in the environment (Stalder et al., 2012). The class 1 mobile integrons are present in high concentrations in polluted water. Thus, they are found in WWTPs in concentrations ranging within  $10^{10}$  - $10^{12}$  copies per litre in effluents (Stalder et al., 2012). Integrons are present in virtually all stages of treatment in WWTPs (Stalder et al., 2012). Significant concentrations of about 40% integrons belonging to class 1 integrons have been found in activated sludge (Stalder et al., 2012). However, class 2 and class 3 integrons have been identified in WWTPs in concentrations much lower than that of class 1 integrons (Stalder et al., 2012).

Integrons of medical interest belonging mostly to the class 1 integrons have been widely studied (Stalder et al., 2012). These studies were mainly concerned in the role of integrons in multiple resistance to antibiotic as well as their relationship with genetic elements such as transposons and plasmids (Moura et al., 2012). Integrons are studied in recent times in wastewater environment, rivers among others to investigate their ecology, diversity etc (Moura et al., 2012).

Distinct studies done on integrons in wastewater environment has detected the abundance of mostly class 1 integrons in wastewater. These integrons are seen to exhibiting resistance to antibiotics most of which are multiple resistance, for instance the work done by Moura et al. (2012) on isolates of bacteria belonging to Enterobacteriaceae and Aeromonas species. Samples obtained from municipal WWTPs revealed the presence of both class 1 and class 2 integrons most of which were chromosomally located. Class 1 integrons was of a higher concentration with most of the identified integrons belonging to the Aeromonas species (Moura et al., 2012). Antibiotic resistant phenotype was exhibited by a significant number of class 1 integrons of which about 80% were multiple resistance. Resistance to antibiotics such as quinolones, erythromycin, ampicillin and cephalothin were exhibited by the integrons (Moura et al., 2012). Also, the transfer of class 1 integron from both Enterobacteriaceae and Aeromonas to recipient *E. coli* was performed, yielding a Conjugation frequency of  $10^{-5}$  and  $10^{-6}$  for Aeromonas and Enterobacteriaceae respectively (Moura et al., 2012).

Furthermore, the study carried out by Kristiansson et al. (2011) on upstream and downstream samples of a WWTP, receiving production water from over 90 drug



manufacturing industries displayed the presence of abundant resistant genes. Concentrations of the resistant genes were higher in downstream compared to upstream. Most of the resistant genes encoded resistance to antibiotics such as sulphonamides, aminoglycoside and fluoroquinolones (Kristiansson et al., 2011). Mobile genetic elements such as class 1 integrons, plasmids and transposons were equally detected in higher concentrations downstream. Carrying resistance genes such as *sul2*, *strA*, *strB* in all the identified plasmids (Kristiansson et al., 2011).

Also, the study performed by Ma et al. (2013) discovered class 1 integron as the most abundant mobile integron in wastewater with some of the integrons encoding multiple antibiotic resistance.

Molecular analysis carried out on gene cassettes of integrons isolated from wastewater, as well as on genes lodged in the gene cassette revealed the presence of ARGs (Stalder et al., 2012). Genes encoding resistance to distinct antibiotics such as aminoglycoside, erythromycin, trimethoprim, quinolones,  $\beta$ -lactams, chloramphenicol and rifampicin were carried in the gene cassettes (Stalder et al., 2012). Analysis of the gene cassette revealed diverse gene cassettes present in wastewater encoding both unknown function and metabolic function (Stalder et al., 2012).

Gene cassettes were identified in the work done by Moura et al. (2012) of which some were novel, found in the class 1 integrons. The gene cassettes identified belonged mainly to the *aadA* cassette which encodes resistance to streptomycin and spectinomycin. Several novel gene cassettes have also been discovered in studies (Tennstedt, Szczepanowski, Braun, Pühler, & Schlüter, 2003).

Integrons are unable to transfer themselves so that their association with other mobile genetic elements such as transposons and plasmids in wastewater, enhances their horizontal transfer in the environment (Ma et al., 2013; Moura, Henriques, Ribeiro, & Correia, 2007). The study performed by Moura et al. (2007) and Tennstedt et al. (2003) revealed the presence of integrons carrying resistant genes on plasmids isolated from bacteria of WWTP. Most of the integrons identified belonged to class 1 and a few class 2 integrons were identified in Moura et al. (2007). Some of the plasmids harbouring integrons carried resistant genes and belonged to the conjugative broad host range plasmids (Tennstedt et al., 2003).

Integrations have also been detected on transposons in gram-negative bacteria (Liebert, Hall, & Summers, 1999). The transposon Tn21 is seen to carry integron in bacteria belonging mainly to Enterobacteriaceae species, such that Tn21 transposons are responsible for multiple resistance in this species (Liebert et al., 1999).

To sum up, integrons are well distributed and abundant in wastewater environment. Most of which are seen to exhibit multiple antibiotic resistance phenotype. Their presence in effluent has shown that the conventional treatment techniques employed are unable to adequately remove both bacteria carrying integrons as well as antibiotic resistant determinants. Thus, the association of integrons on genetic elements such as plasmids and transposons is of great concern in wastewater, as such association can intensify the spread of resistant integrons in the environment.

### 6.5 Plasmid

Plasmids are small circular DNA molecules present in a bacteria cell, capable of replicating autonomously (Bennett, 2008; Cano & Colomé, 1988). Replication in plasmid is rapid owing to the small DNA size they possess (Madigan et al., 2006). The size of plasmids varies ranging from a few base pairs to over kilobases of base pairs (Lodish et al., 2000). Plasmids can carry genes within the range of 2- 3 to hundreds of genes (Bennett, 2008; Madigan et al., 2006).

The genes carried by plasmids are not necessarily relevant for the basic functioning of a bacterial cell (Bennett, 2008) but can be of benefit to host cell in terms of adaptation etc (Lodish et al., 2000). Functions of genes carried in plasmids includes, genes encoding resistance to antibiotics, genes conferring resistance to heavy metals such as cadmium and mercury, metabolic function, genes encoding for conjugation etc (Bennett, 2008; Cano & Colomé, 1988; Madigan et al., 2006). Plasmids carrying genes encoding resistance to antibiotics are termed resistant plasmids (R- plasmids), most of which are self-transmissible conjugative plasmids (Bennett, 2008). Therefore, plasmids have been extensively studied in terms of conferring resistance (Brown-Jaque et al., 2018) and have been seen to confer antibiotic resistance to bacteria through the process of conjugation following their discovery in the 1950s (Madigan et al., 2006).

Different types of plasmids can be present in a bacterium cell with their numbers varying. The numbers of plasmid present in a given bacterium cell is referred to as its copy number and this number is usually regulated by genes carried in the plasmid (Madigan et al., 2006).

Plasmids can either transfer themselves from one bacteria to the other or be mobilized and transferred by other self-transmissible plasmids (Bennett, 2008). The transfer of non-transmissible plasmids is achieved through the process of conjugation by self-transmissible plasmids such as conjugative plasmids (Bennett, 2008). Conjugative plasmids are further categorized into broad host range and narrow host range, depending on the number of bacteria species they can transfer to (Bennett, 2008).

Plasmids are classified into Incompatibility groups based on their mechanism of replication and partitioning (Popowska & Krawczyk-Balska, 2013). Plasmid incompatibility group ranges from IncA to IncZ with IncP, IncW, IncN and IncQ groups seen to convey antibiotic resistance, as well as exhibit broad host range (Popowska & Krawczyk-Balska, 2013). Plasmids belonging to the same incompatibility group are genetically similar in that they replicate or partition via a similar mechanism (Popowska & Krawczyk-Balska, 2013). Thus, plasmids of the same incompatibility group cannot exist in the same bacteria, as one of such plasmid is lost from the cell in the course of cell replication (Madigan et al., 2006).

The presence of plasmids on bacteria population in different environment and the ability of conjugative resistance plasmids to exhibit broad host range, has resulted to plasmid being studied in wastewater environment as vectors of antibiotic resistant determinants.

Conjugative resistance plasmids belonging mostly to the IncP group were identified in the work done by Dröge, Pühler, and Selbitschka (2000), on plasmids from bacteria isolated from a municipal WWTP. Transfer of the distinct identified plasmids was carried out from *Pseudomonas* to *E. coli*, *E. coli* to *Pseudomonas* and *Pseudomonas* to *Sinorhizobium meliloti*. Conjugative frequencies within the ranges of  $< 3.3 \times 10^{-9}$  to  $8.0 \times 10^{-2}$ ,  $1.2 \times 10^{-8}$  to  $8.6 \times 10^{-1}$  and  $1.2 \times 10^{-1}$  to  $< 8.3 \times 10^8$  were obtained respectively, with most of the IncP plasmids exhibiting very high frequencies within  $10^{-1} - 10^{-2}$  per recipient cell (Dröge et al., 2000). Furthermore, virtually all the IncP plasmids conferred multiple resistance on host bacteria when tested using antibiotics at minimal inhibitory concentration (Dröge et al., 2000).

The study performed by Schlüter, Krause, Szczepanowski, Goesmann, and Pühler (2008), discovered plasmids encoding antibiotic resistance and virulence traits by sequencing plasmids from bacteria of WWTPs. Resistant plasmids identified encoded resistance mostly for b-lactam antibiotic. Mobility of the identified plasmids was determined, as most of them were mobilizable plasmids possessing genes capable of mostly relaxosome and DNA-processing for transfer function (Schlüter et al., 2008). Nevertheless, a few conjugative plasmids belonging to IncF and IncX compatibility group were discovered. Also the presence of transposable elements such as insertion sequence and transposons was discovered on some plasmids, with some transposons conveying resistance to both antibiotics and metals (Schlüter et al., 2008).

Furthermore, in the study done by Stefan and Soud (2017), Plasmids belonging to the incompatibility group IncP, IncF, IncW and IncQ were discovered in samples from influent and effluent of a WWTP. Conjugative transfer was performed from a donor *E. coli* cell carrying exogenous IncP plasmid, to recipient bacteria cells isolated from the WWTP. Most of the recipient bacteria belonged mostly to Proteobacteria, Firmicutes, Bacteroidetes. Higher conjugation frequency was recorded in the bacteria cells isolated from influent and high diversity of transconjugants was seen in the effluent as well as high recipient diversity (Stefan & Soud, 2017). Thus, indicating that the process of conjugation occurs more in the influent of WWTP, which may be due to the presence of high cell density (Stefan & Soud, 2017).

Lastly, with the presence of high diversity of transconjugants in effluent (Stefan & Soud, 2017), it can be inferred that the process of conjugation is likely to occur in the receiving environments such as rivers, streams etc at a relatively high rate than expected.

## 6.6 Transposon

Transposons are small DNA segments capable of integrating into chromosome of bacteria (Cano & Colomé, 1988). They belong to the group of transposable elements of which insertion sequence (IS) is a member (Bennett, 2008; Madigan et al., 2006). This group of transposable elements that is transposon and IS exhibit certain similarities, in that they both possess inverted repeats at their ends as well as the transposase enzyme which enables the process of transposition (Madigan et al., 2006). Transposons differ from IS in that they are larger and carry several genes encoding both the transposase protein and antibiotic

resistance (Madigan et al., 2006). Consequently, resulting in transposons being able to convey resistance phenotype to its host bacteria (Bennett, 2008). On the contrary, insertion sequence only carry the gene encoding the transposase and as such do not confer antibiotic resistance to their host cell (Madigan et al., 2006).

Insertion sequence as well as transposons can be found in the plasmids and chromosomes of bacteria (Cano & Colomé, 1988). Transposons are capable of integrating themselves at different locations on the chromosome of a bacteria so that they are referred to as jumping genes (Cano & Colomé, 1988). Transposons carrying resistance genes are termed Resistance transposons (Bennett, 2008), they exist in distinct forms and differ in their process of transposition as well as in structure (Bennett, 2008). Examples of resistance transposons include Tn5 encoding resistance to aminoglycosides, Tn3 encoding resistance to b-lactam, Tn10 conferring resistance to tetracycline and T21 encoding resistance to streptomycin, spectinomycin and sulphonamides etc (Bennett, 2008).

The presence of transposase protein and repeat ends in transposable elements are of great importance in the process of transposition (Madigan et al., 2006). Transposition is the movement of genetic material from one location to another, it is a site specific recombination process requiring the presence of inverted ends (Madigan et al., 2006). Transposition occur in two ways that is conservative transposition and replicative transposition based on the mechanism involved (Madigan et al., 2006). In conservative transposition, transposons are removed and inserted in to new sites. Although in replicative transposition copies of transposons are inserted into new sites (Madigan et al., 2006).

Conjugative transposons are able to transfer themselves from one bacteria to another (Iyer et al., 2013), so that they are able to transfer resistance when they carry the genes. Resistance conferred by transposons is said to have a positive effect on bacteria cell fitness, as such bacteria is likely to withstand selective pressure (Iyer et al., 2013).

Studies has discovered the presence of transposons conveying resistance in wastewater environment (Schlüter et al., 2008; Szczepanowski et al., 2004). Furthermore, the presence of *E. coli* in wastewater which is known to harbour significant number of resistant transposons such as Tn3, Tn7, Tn9, Tn10 among others (Iyer et al., 2013). In addition to

association of integrons with transposons, indicates that transposons play roles in the dissemination of resistant determinants in wastewater.

## Conclusion

Studies has confirmed that antibiotics are present in wastewater environment, owing to the indiscriminate and extensive use of antibiotics. Antibiotics such as quinolones, macrolides, sulfonamides, tetracycline, erythromycin etc occur recurrently in WWTPs globally. Wastewater habitats such as WWTPs contain antibiotics in different degrees. Concentrations were seen to be high in developing countries compared to those obtained in developed countries. This may have been because of the unregulated use of antibiotics in developing regions. It has also been seen that wastewater harbours resistant determinants such as ARGs and ARBs, as conditions favouring their development that is high cell density and selective pressure are prevalent. Furthermore, resistance phenotype exhibited by bacteria is either acquired or inherent. Despite the type of resistance present in a bacterium, several mechanisms which is either molecular or genetically based is employed by bacteria to exhibit resistance. Resistance arising from the distinct mechanism are mostly due to alteration of target site, reduced affinity of antibiotic molecule to target site as well as extrusion of the antibiotic. Bacterial community in wastewater carry mobile elements such as bacteriophages, plasmids, integrons and transposons, which can acquire and transfer both resistant and non- resistant genes among bacteria. Resistance genes such as bla<sub>TEM</sub>, bla<sub>CTX-M</sub>, strA, strB, qnrA are carried by mobile element. Plasmids belonging mostly to IncP, class 1 integrons, and *E. coli* phages are significantly present in wastewater, with most of them exhibiting broad host-range as well as being conjugative. They are also known to carry multiple resistance particularly class 1 integrons because of the gene cassettes they possess. However, there is still no data on the actual pattern through which resistance genes are spread in wastewater systems as well as their rates of spreading. Knowledge on both patterns and rates of ARGs spreading will be of importance in reducing their presence in the environment. Also, the fate of ARGs in wastewater and in receiving domains of wastewater such as river is not known. Furthermore, we cannot attribute the distinct antibiotic resistance exhibited by wastewater bacteria to a specific source. That is if the resistance is due to antibiotic in waste water or it was acquired from other environments such as hospital. Studies performed in vitro with isolated resistant mobile elements from wastewater, showed that resistance genes are transferred. What we do not know is if the transfer of resistance genes in wastewater increases the resistome of the domain. Thus,

mobile genetic elements are carriers of resistance genes and their presence in wastewater spreads resistance in the domain.

#### Future Perspectives

Study on environments receiving wastewater such as river, to know if they pose a threat to the biota and fauna present. If they do, what kind of threat. This will provide data on the kind of risks ARGs can pose when released to the environment.

Study using either models, biosensors, or gene construct to determine if these genes are spread. Knowledge in this area will contribute to know the rates at which genes spread



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