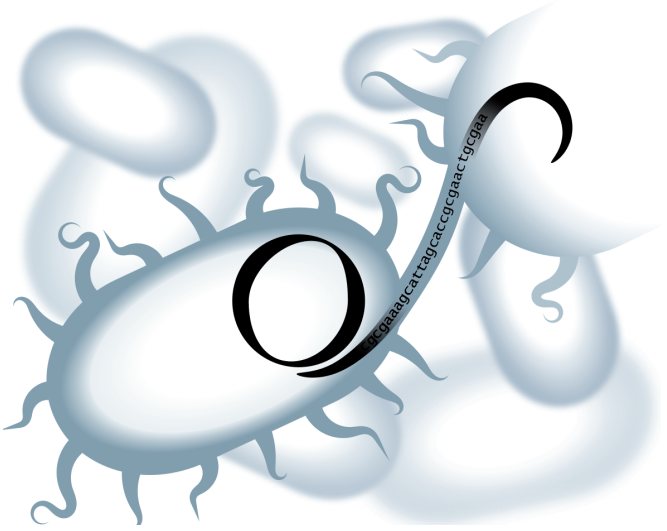
Fluoroquinolone resistance in the environment and the human gut

Analysis of bacterial DNA sequences to explore the underlying genetic mechanisms



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We're not scaremongering
This is really happening

Abstract

Fluoroquinolones (FQs) are synthetic, broad-spectrum antibiotics that target type II topoisomerases. High-level resistance is often caused by mutations in the target genes of FQs, especially in *gyrA* and *parC*. In contrast, plasmid-mediated resistance genes, such as *qnr*, often confer moderate levels of resistance. Several sites near Patancheru, India, have been previously shown to be severely contaminated with FQs. To study how environmental bacteria adapt to this extreme environment, we first used whole-genome sequencing (454) of a highly multi-drug resistant strain of *Ochrobactrum intermedium*. The strain was isolated from a wastewater treatment plant (WWTP) in Patancheru that treats industrial effluent from pharmaceutical production. The strain was considerably more resistant to tetracyclines, sulphonamides, and FQs than to other *O. intermedium* strains, and it had, accordingly, acquired a tetracycline efflux pump, a sulphonamide resistance gene, and mutations in the target genes for FQs. In the second study, sequencing (Illumina) was used to characterise horizontally transferrable resistance plasmids captured from bacterial communities sampled from a lake with a history of FQ pollution, near Patancheru. All transconjugants had acquired *qnr* genes and this is, to the best of our knowledge, the first time *qnrVC1* has been described on a conjugative plasmid. Furthermore, the bacteria from the lake sediments were significantly more resistant to FQs and sulphonamides compared to bacteria from Indian and Swedish reference lakes.

In the third study, the *Escherichia* communities inhabiting a stream in Patancheru receiving WWTP effluent with high levels of FQs were tested for resistance mutations in *gyrA* and *parC* using amplicon sequencing (454). A stream receiving municipal WWTP effluent in Skövde, Sweden, and a remote highland lake were included as references. To our surprise, all communities showed high abundances of FQ resistance mutations, suggesting that these mutations are not associated with a fitness cost in the studied environments. The same method was utilised in the fourth study, on faecal samples collected from Swedish students before and after travel to India. The abundance of the amino acid substitution S83L in GyrA increased significantly, and the number of observed genotypes decreased after travel. This finding shows that international travel contributes to the spread of bacteria carrying chromosomal resistance mutations. Taken together, the development and spread of antibiotic resistance from antibiotic-polluted environments is a concern for everyone.

Populärvetenskaplig sammanfattning

Antibiotika är läkemedel som används för att förebygga och bota sjukdomar orsakade av bakterier. De är oumbärliga inom modern medicin och möjliggör inte bara behandling av infektioner som t.ex. lunginflammation – som innan antibiotika började användas kunde vara en dödlig sjukdom – utan även organtransplantation och cellgiftsbehandling vid cancer. För att behandlingen skall få önskad effekt krävs att bakterierna är känsliga mot antibiotikumet. Bakterier som är resistenta, alltså motståndskraftiga, kan orsaka långvarig sjukdom vilket ökar risken för att smittan sprids och att patienten avlider. Somliga bakterier är naturligt resistenta mot vissa sorters antibiotika, men även känsliga bakterier kan bli resistenta genom att de gener de bär på muterar, eller genom att de tar emot resistensgener från en annan bakterie. Om stora mängder antibiotika konsumeras, eller om antibiotika förskrivs i felaktiga doser, kan andelen resistenta bakterier öka eftersom de känsliga bakterierna dör medan de resistenta frodas. Runt om i världen kämpar man allt mer mot bakterieinfektioner som inte går att bota på grund av antibiotikaresistens. WHO har därför omnämnt den ökande resistensen som en global nödsituation för människors hälsa.

Många antibiotika är ämnen som produceras naturligt av mikroorganismer. Alexander Flemings upptäckt att mögelsvampen *Penicillium rubens* producerar ett ämne som dödar bakterier, penicillin, gav honom Nobelpriset och blev startskottet för den antibiotiska eran. Antibiotika finns naturligt i miljön i låga koncentrationer och följaktligen också bakterier som bär på resistensgener som skyddar organismen mot antibiotika. Substanserna kan även hamna i miljön via oss människor samt våra husdjur och boskap. Under en behandling passerar en del av läkemedlet kroppen oförändrat och utsöndras i urin eller avföring. Reningsverken är sällan anpassade för att bryta ner antibiotika och vissa stabila sorter kommer därför ta sig genom reningsprocessen och ut i naturen. Vad forskare inom området oroar sig för är om reningsverk och andra platser som har förhöjda koncentrationer av antibiotika utgör miljöer där resistenta miljöbakterier trivs. Detta skulle då öka risken för att de för över sina resistensgener till sjukdomsalstrande bakterier – som t.ex. kan komma till reningsverket via hushållsavlopp – och på så sätt skapar motståndskraftiga bakterier som är farliga för människan. På detta sätt kan även nya gener, som vi ännu inte träffat på i sjukhusen, spridas från miljön in till kliniken. Vilka koncentrationer av antibiotika som krävs för att de resistenta bakterierna skall öka i

andel, och hur stor risken för genöverföring till sjukdomsalstrande bakterier är, behöver dock studeras i mer detalj.

I denna avhandling har resistens mot en särskild grupp av antibiotika undersökts: kinoloner. De har till skillnad från många andra antibiotika inte ett naturligt ursprung utan är helt konstgjorda substanser, och är därför inte är vanliga i naturen. Vi har studerat dels vattenmiljöer kring Patancheru i Indien, svårt förorenade av kinoloner genom utsläpp från läkemedelsindustri, och dels svenska vattendrag där inga kinoloner uppmätts. Till vår förvåning var andelen som bar på resistensmutationer i de studerade bakteriesamhällena hög i både de rena och de förorenade miljöerna. Detta tyder på att dessa mutationer inte gör det svårare för bakterien att överleva jämfört med omuterade bakterier, vilket skulle kunna förklara varför bakterier kan bli kinolonresistenta så snabbt. Samma antydan fanns när vi undersökte förekomsten av samma mutationer i tarmfloran hos svenska studenter. Eftersom deltagarna hade lämnat prover både innan och efter att de rest till Indien för att studera eller praktisera, kunde vi även visa att andelen studerade bakterier med resistensmutationer ökade efter resan. Vi kunde dessutom se tecken på att mångfalden i den delen av tarmfloran vi undersökte minskade under resan.

Från ett reningsverke i Patancheru som tar emot spillvatten från antibiotika-producenter i regionen, isolerade vi en miljöbakterie som var resistent mot en stor majoritet av alla sorter av antibiotika. Genom att jämföra dess arvsanlag med det från en känsligare bakterie av samma art kunde vi fastställa hur den indiska bakteriestammen förvärvat sin resistens. De mutationer och resistensgener som den multiresistenta bakterien bar på gjorde det möjligt för den att överleva i den förorenade miljön i reningsverket. Andra kontaminerade miljöer vi studerade i denna avhandling innefattade sjöar nära Patancheru som har visats vara svårt förorenade av bland annat kinoloner. Vi visade att bakteriesamhällen från dessa sjöar utgjordes av en större andel resistenta bakterier jämfört med baterier från andra sjöar i regionen som inte varit lika förorenade och rena svenska sjöar. Dessutom kunde vi klarlägga att denna motståndskraft gick att överföra till *E. coli*, en bakterieart som är vanliga i vår tarm och som ibland orsakar svåra infektioner. Både studien av bakteriestammen från reningsverket och studien av de kontaminerade sjöarna tyder på att det finns miljöer där koncentrationen av antibiotika är tillräckligt hög för att gynna resistenta bakterier. Sannolikt kan även betydligt lägre koncentrationer räcka för att främja dessa bakterier, men det behövs ytterligare studier för att få en fullständig uppfattning om riskerna. I dagens värld med global handel och förflyttning av både varor och människor spelar det i princip ingen roll var motståndskraftiga bakterier uppstår. De sprids snabbt mellan länder och därför är det ökade hotet från antibiotikaresistenta bakterier en global angelägenhet.

List of Papers

This thesis is based on the following articles and manuscripts:

Paper I **The acquired genetic mechanisms of a multi-resistant bacterium isolated from a treatment plant receiving wastewater from antibiotic production**
Anna Johnning, Edward R. Moore, Liselott Svensson-Stadler, Yogesh S. Shouche, D. G. Joakim Larsson & Erik Kristiansson
Applied and Environmental Microbiology 79.23 (2013): 7256-7263.

Paper II **Isolation of novel broad host fluoroquinolone resistance plasmids from an antibiotic-polluted lake**
Carl-Fredrik Flach, Anna Johnning, Ida Nilsson, Kornelia Smalla, Erik Kristiansson & D. G. Joakim Larsson
Manuscript

Paper III **High abundance of resistance mutations in *gyrA* and *parC* in bacterial communities sampled in both fluoroquinolone polluted and pristine environments**
Anna Johnning, Erik Kristiansson, Jerker Fick, Birgitta Weijdegård & D.G. Joakim Larsson
Submitted

Paper IV **International travel affects the abundance of chromosomal quinolone resistance mutations in human gut microbiome**
Anna Johnning, Erik Kristiansson, Martin Angelin, Nachiket Marathe, Yogesh S. Shouche, Anders Johansson & D.G. Joakim Larsson
Submitted

Paper not included in the thesis:

A novel method to discover fluoroquinolone antibiotic resistance (*qnr*) genes in fragmented nucleotide sequences
Fredrik Boulund, Anna Johnning, Mariana B. Pereira, D.G. Joakim Larsson & Erik Kristiansson
BMC Genomics 13.1 (2012): 695.

Content

Introduction

Antibiotics

The introduction of antibiotics in the early 1940s revolutionised clinical practice, and these drugs have become indispensable in modern medicine. It is difficult to picture a world with no effective antibiotics, as they are not only used to fight bacterial infections but also to prevent infections in connection with surgery, organ transplantation, and cancer chemotherapy[1]. Indeed, three different Nobel prizes in physiology or medicine have been rewarded for discoveries of antibiotic substances, signifying the importance of these compounds[2].

Here, the term antibiotic is used for all compounds utilised against bacterial infections, and they can be divided into different classes according to the processes they target within the bacterial cell. The β-lactams (carbapenems, cephalosporins, and penicillins), for example, target cell wall synthesis; sulphonamides and trimethoprim inhibits folic acid synthesis; aminoglycosides, chloramphenicol, tetracyclines, and macrolides interfere with protein synthesis; and quinolones target the management of DNA topology (i.e., the supercoiling of the circular DNA strand)[3]. Antibiotics can also be divided into groups depending on their origins. Some are entirely natural compounds produced by microorganisms, some have a natural substance as the starting compound in their chemical synthesis and are, therefore, semi-synthetic, and others are entirely man-made.

The European Centre for Disease Prevention and Control (ECDC) monitors the consumption of antibiotics in the primary care and hospital sectors in the European Union (EU), Iceland, and Norway. In 2011, the median consumption of antibacterials for systemic use in the community was 19.5 Defined Daily Doses (DDD) per 1 000 inhabitants and day[4]. Cyprus had the highest consumption with 35.1 DDD per day and 1 000 inhabitants, while the Netherlands had the lowest with 11.4. Sweden had the sixth lowest consumption with 14.3 DDD per day and 1 000 inhabitants. However, the majority of antibiotics consumed in the world are administered to animals, including livestock and companion animals[5]. In contrast to human care, animal husbandry often treats large groups of animals at the same time, not only to treat disease but also as for prophylaxis or growth promotion. In

the 25 European countries reporting to the European Medicines Agency (EMA), 8 481 tonnes of active antibiotic ingredient were consumed in 2011[6].

Antibiotic Resistance

Not long after the therapeutic use of antibiotics began, resistant bacterial infections emerged that did not respond to treatment. Indeed, only a few years after penicillin was introduced, the first resistant *Staphylococcus aureus* infections were discovered, and in 1946, 60% of *S. aureus* isolated in British hospitals was already penicillin resistant[7]. Resistant bacteria cause refractory infections that lead to prolonged illness and increase the risk of transmission to others and death. Decades of extensive and sometimes redundant consumption of antibiotics all over the world have led to the promotion of resistance. In Europe, the ECDC collects monitoring data on antibiotic resistance from invasive isolates drawn from blood and cerebrospinal fluid[8]. During 2012, the majority of all reported *Escherichia coli* isolates were resistant to at least one of the tested antibiotics, and only 39.5% of them were fully susceptible to aminopenicillins, fluoroquinolones, third-generation cephalosporins, aminoglycosides, and carbapenems. Finland had the highest proportion of fully susceptible isolates (57.2%) and Bulgaria had the lowest proportion (22.3%).

International travel, migration, and the global market for goods and food have greatly contributed to the rapid spread of resistant bacteria[9]. The human gut microbiota has been shown to contain a greater abundance of antibiotic-resistant bacteria after international travel to countries with a higher resistance burden, as indicated by culturing experiments[10, 11]. Furthermore, PCR has been used to demonstrate that selected mobile antibiotic resistance genes increase in abundance in the microbiota after travel[12-14]. However, changes in chromosomal resistance mutations related to travel have not been investigated.

The major mechanisms of antibiotic resistance include inactivation of the drug, alteration, protection or replacement of the target, and decreased intracellular concentration of the drug[3]. Examples of drug inactivation mechanisms include β-lactamases, which are enzymes able to disrupt the β-lactam ring found in cephalosporins, carbapenems, and penicillins[15]. Other bacterial enzymes are capable of reducing the effect of a drug by introducing a functional group to the antibiotic, for example, acetylation of aminoglycosides. Alternatively, the drug target can be modified through mutations so that its affinity to the drug is lowered, which is the case for certain amino acid substitutions in the penicillin-binding proteins. Furthermore, the target can be protected from the drug, as in the enzymatic methylation of the ribosomal rRNA in the 23S subunit that confers macrolide resistance. The bacterium can also acquire genes with the same function

as the target gene that are unaffected by the drug, for example, a new dihydropteroate synthase stable against sulphonamides. Finally, efflux pumps and alteration of cellular drug uptake can reduce the levels of the antibiotic in a bacterium to ineffective concentrations.

Some bacteria are intrinsically resistant, but a sensitive bacterium can gain resistance – caused by any of the general mechanisms – through mutations in existing DNA or the acquisition of resistance genes by horizontal gene transfer (HGT)[3]. HGT allows bacteria to share genetic content, even between species, and it is one of the reasons that antibiotic resistance can spread rapidly in a bacterial population[16]. Conjugation is one method of HGT by which antibiotic resistance genes are acquired. This mechanism involves the transfer and replication of a mobile genetic element – such as plasmids and integrative genetic elements (ICEs)[17] – from one cell to another. A genetic element is said to be conjugative if it contains the genes necessary for the entire transfer process: (1) a mobility module (MOB) containing an origin of transfer (*oriT*), and genes encoding a relaxase and a type IV coupling protein (T4CP); and (2) genes encoding a type IV secretion system known as mating pair formation (MPF) complex. The MOB genes are responsible for initiation and execution of the transfer while the MPF genes are responsible for the channel through which the DNA strand is transported. When a plasmid carries only the MOB and utilises another element's MPF complex, it is classified as mobilisable. Conjugation contributes to the plasticity of bacterial genomes, but the content of the plasmids themselves can also be flexible. Integrons, for examples, can be found on plasmids and are elements that capture mobile genes – such as antibiotic resistance genes – into their gene cassettes by recombination and then express them[16]. These elements consist of a gene encoding an integrase (*intI*), a recombination site (*attI*), and promoters that control the expression of all the gene cassettes[18]. Integrons can also be found on transposons, another mobile genetic element know to carry antibiotic resistance genes[16].

Fluoroquinolones and Resistance

Quinolones are a synthetic class of antibiotics introduced to the clinical and veterinary medicine in the 1960s[19]. They can be divided into generations that are loosely based on their bacterial spectra[20]. The first generation of quinolones was active only against Gram-negative bacteria, but the addition of a fluorine atom at position six in the central carbon ring system – creating the fluoroquinolones – increased the antibacterial spectrum greatly. Ciprofloxacin, which was approved for clinical use in the end of the 1980s, is an example of a second-generation quinolone. Due to their broad-spectrum activity, fluoroquinolones are used to treat a number of different human and animal infections. Indeed, in 1997, ciprofloxacin was

reported as the most used antibiotic in the world[21]. In the 29 European countries reporting their antibiotic consumption to ECDC in 2011, quinolones were the third most sold class of antibiotic[4]. The median consumption was 1.29 DDD per 1 000 inhabitants and day, and Cyprus reported the highest numbers (3.8 DDD), while the United Kingdom reported the lowest consumption (0.43 DDD). A majority of the sold quinolones are of the second generation, with ciprofloxacin constituting 73% of the sales in all countries. With 0.43 DDD per 1 000 inhabitants and day, Sweden had the fourth lowest consumption of the monitored countries and also showed a significant decrease in quinolone sales during the five-year period ending in 2011. Surveillance outside of Europe is less systematic, but there have been reports from regions with higher consumption. In New Delhi, India, for example, four wards monitored their antibiotic consumption during 2008 and found that 33% of the DDD of antibiotics sold at private clinics were fluoroquinolones (74 DDD per 1 000 patients and day)[22]. In Sweden, only 5.4% of the consumed antibiotics for systemic use in 2011 were fluoroquinolones[4]. Fluoroquinolones are also used to treat animals, although the use is forbidden or highly restricted in some parts of the world[5]. The sale of antibiotics to food producing animals in Europe during 2011 varied between nations: between 14.0% and 0.3% in Slovenia and in Denmark, respectively[6]. Surveillance data from developing countries are scarce, but a report from Kenya that covered 1995-1998 indicated that, on average, 0.64% of antibiotics administered to food-producing animals were quinolones[23].

Quinolones target type II topoisomerases, DNA gyrase and topoisomerase IV, which manage DNA topology. These enzymes catalyse the negative supercoiling of DNA – necessary in DNA replication and transcription – by cleaving double-stranded DNA to allow another DNA strand to pass[24]. The drug binds to the DNA-enzyme complex and stabilises the cleaved form, thereby, inhibiting the re-ligation of the DNA strand[25]. In doing so, quinolones prevent DNA replication, leading to bacteriostasis; the mechanism for the bactericidal effect of the drugs is more complex[25, 26]. In Gram-negative bacteria, DNA gyrase is the primary target of the antibiotics, and topoisomerase IV is the main target in Gram positives.

Alterations to the targeted enzymes are an important cause of high-level fluoroquinolone resistance. Both enzymes are tetrameric (X_2Y_2), and DNA gyrase is encoded by the genes *gyrA* and *gyrB*, while topoisomerase IV is encoded by the genes *parC* and *parE*. The enzymes are homologous with a considerable sequence similarity between *gyrA* and *parC*, as well as between *gyrB* and *parE*. Most of the known mutations that have been associated with resistance are found within the quinolone resistance determining region (QRDR), which is defined as codon 67-106 in *gyrA* and 426-447 in *gyrB*[24]. When the homologous proteins are aligninged, the corresponding codons are 64-103 in *parC* and 420-441 in *parE*. There are

additional mutations outside of the QRDRs that have been linked to reduced fluoroquinolone susceptibility, especially in *parE*[24]. In addition to target alterations, bacteria can acquire mobile plasmid-mediated quinolone resistance (PMQR) genes. The first transferable quinolone resistance was reported in 1998[27]; before this discovery, it was believed that PMQR would be unlikely. Antibiotic resistance genes were argued to originate in antibiotic-producing microorganisms, and because quinolones are synthetic compounds, there would have been no need for transferable resistance to evolve[28]. The discovery of the first PMQR gene, hence, took researchers by surprise. The gene – which was named *qnr* – protects the target enzymes from quinolone binding, and since then more versions have been described[29]. The acquisition of a *qnr* gene generally provides moderate levels of and can increase the minimum inhibitory concentration (MIC) of ciprofloxacin to 2.0 mg/L, but, more commonly, it increases to approximately 0.25 mg/L. The *aac(6')-lb-cr* gene is less protective than the *qnr* genes (ciprofloxacin MIC up to 0.008 mg /L)[30], and its mechanism involves the acetylation of ciprofloxacin and norfloxacin. There are also both chromosomal and plasmid-mediated effluent pumps that are able to lower the intracellular concentration of quinolones.

According to European Committee on Antimicrobial Susceptibility Testing (EUCAST), an Enterobacteriaceae infection is classified as clinically resistant to ciprofloxacin if the MIC is higher than 1 mg/L and sensitive if it is 0.5 mg/L or less[31]. In a study of 78 *Escherichia coli* isolates collected at two hospitals in Houston, Texas, USA, all fluoroquinolone-resistant strains had at least one amino acid substitution in GyrA, and 85% had additional substitutions in ParC[32]. None of the tested strains carried *qnrA,* but 25% were *aac(6')-lb-cr* positive; all of the *aac(6')-lb-cr* positive strains had at least three mutations in the target enzymes. Mutations were also detected in *gyrB* and *parC*, but they did not appear to affect the MICs for the tested fluoroquinolones. Indeed, it has been demonstrated that *E. coli* has to acquire at least three mutations in the target enzyme, two of which must be in gyrA, to gain clinical resistance to fluoroquinolones[33]. However, strains with identical substitutions can have greater than10-fold differences in MIC[32]. In the European countries reporting to ECDC, 22.3% of the invasive *E. coli* infections were classified as fluoroquinolone resistant in 2012[8]. Cyprus and Italy had the highest percentage of resistant isolates (42.0%), Iceland had the lowest (9.7%), and had Sweden the second lowest (11.2%). A trend analysis over the five-year period ending in 2012 revealed a significant increase in resistance in ten countries, including Sweden, and a decrease in only two. In India, with considerably higher fluoroquinolone consumption, one hospital reported that 73% of urinary tract infections caused by *E. coli* were ciprofloxacin resistant in 2008[34].

Antibiotics and Antibiotic Resistance in the Environment

Because many antibiotics are natural compounds, antibiotic resistance is also prevalent in nature and existed long before humans started using antibiotics for therapeutic purposes. Indeed, antibiotic-resistant bacteria have been isolated from inside a remote cave system, estimated to have been isolated for millions of years[35], and antibiotic resistance genes have been detected in 30 000-year-old permafrost[36]. Furthermore, soil bacteria have even been reported to be culturable with antibiotics as their only carbon source[37]. This was shown for antibiotics belonging to different classes and representing natural, semi-synthetic, and synthetic compounds. The collection of all resistance determinant, and determinants with the potential to evolve into resistance factors, that are present in bacteria has been labelled the resistome[38]. The resistance factors faced in the clinical setting are likely only a small fraction of the entire resistome, and the environment could serve as a reservoir of novel resistance genes[37].

The ubiquitous presence of resistance determinants in nature has raised concerns about anthropogenic pollution by antibiotics in the environment[39]. Antibiotic pollution originates from multiple sources and reaches the environment via different pathways[40-42]. For example, improper disposal of unused drugs occurs, and when humans and animals are treated, a fraction of the drug gets excreted un-metabolised. If the active ingredient is not rapidly degraded, it might end up in the soil or in the aquatic environment, often via wastewater treatment plants (WWTPs) or landfills. In WWTPs, antibiotics that pass through the treatment process will either be released with the effluent or end up in the sludge. The typical concentrations of antibiotics detected in the environment are low. In WWTP effluent, the concentration is usually below 6 μg/L[43], in surface water it is even lower (up to 2 μg/L[43]), and antibiotics are rarely detected at all in drinking and ground water (up to 0.5 ng/L[43])[44]. However, direct emissions from the manufacture of antibiotics have led to considerably higher environmental concentrations. Concentrations reaching the mg/L-range have been detected in industrial effluents, as well as in surface water contaminated by pharmaceutical production[45-47].

The main perceived risk of antibiotic pollution is that sufficiently high concentrations may create selection pressure, promoting resistant bacteria in the environment. Some pathogens are waterborne and naturally reside in the environment; therefore, a selective advantage for resistant pathogens could increase their abundance and, consequently, increase the risk of humans or animals falling ill with refractory infections. However, another risk scenario involves the selection of environmental bacteria that are not necessarily pathogenic. We can assume with high certainty that the environmental resistome includes resistance determinants

not yet encountered in pathogens. If the environmental bacteria carrying "novel" resistance factors gain a selective advantage due to antibiotic pollution, and if this occurs in a setting in which there are also pathogens present, there is an increased risk of the resistance factors being transferred into a pathogen through HGT[48]. Through this pathway, completely new mobile resistance genes could find their way into the clinic. The settings in which the risk of such a transfer is increased – those with a sufficiently high concentration of antibiotics and a mixture of environmental and pathogenic bacteria – are sometimes referred to as "hot-spots". WWTPs that receive human sewage and soils fertilised with WWTP sludge or manure from antibiotic-treated animals, have been highlighted as such hot-spots[48, 49]. What concentrations would promote such selections is largely unclear. However, it is known that in the laboratory setting, resistant bacteria have a selective advantage at an antibiotic concentration several hundred-fold below the MIC[50].

Patancheru in India

For some years, our research group has studied environmental antibiotic pollution in India caused by the improper disposal of waste from drug manufacturing. The large city of Hyderabad in the southeast of India is the centre of a region with a high density of biotechnology and pharmaceutical industries. The Patancheru suburb has an industrial WWTP that receives effluent from approximately 90 bulk drug producers. In the treated effluent leaving the plant, we have previously detected high levels of several pharmaceuticals, including broad-spectrum antibiotics such as fluoroquinolones[46, 47]. Ciprofloxacin was the most abundant drug, and it was detected in the concentration range of 14-31 mg/L. The fluoroquinolones enrofloxacin, norfloxacin, lomefloxacin, enoxacin, and ofloxacin were each detected in concentrations of 0.9-0.1 mg/L. The effluent has been shown to affect a number of different organisms, including bacteria (effects from 3% dilution)[47], plants (1.6-35% dilution)[47], water fleas (6.7-7.2% dilution) [47], tadpoles (0.2% dilution)[51], and fish (in the range of 0.3-7.8% dilution)[51-53]. Together with the illegal dumping of industrial waste, the effluent leaving this WWTP polluted the surface, well, and drinking water, as well as stream sediment in the region with antibiotics[45, 46].

To study the effect on the bacterial communities residing in the water stream receiving the WWTP effluent, we have previously studied sediment samples collected upstream and downstream from the discharge pipe[45]. Additional reference samples were collected from a stream connected to the municipal WWTP in Skövde, Sweden, where no antibiotics were detected in the stream sediment. The abundances of antibiotic resistance genes and genes involved in horizontal gene transfer were detected using metagenomic sequencing. This method enabled an explorative screen of the entire bacterial community for all known genes without

relying on the culturability of the bacteria. The results showed that the antibiotic pollution had promoted a number of different resistance genes at the sampled downstream sites compared to the upstream sites. Large numbers of integrons, transposons, and plasmids were also detected. Furthermore, the treatment plant itself has been shown to be inhabited by highly multi-resistant strains[54]. The screening of 93 strains isolated from different parts of the WWTP showed that 86% were resistant to at least 20 of the 39 tested antibiotics (belonging to 12 different antibiotic classes). Almost all strains (95%) harboured at least one integron of either class 1 or 2. The results indicate that a high environmental antibiotic selection pressure will select for resistant environmental bacteria. Furthermore, the bacteria also carry genetic elements that could mobilise and spread the resistance genes.

Aims

The external environment plays an important role in the emergence and spread of antibiotic-resistant pathogenic bacteria. Over the last decade, environmental antibiotic pollution has been identified as a potential contributor to these processes by selecting for resistant bacteria, increasing the risks for their colonisation of the human flora, and transferring resistance factors to human pathogens. This thesis aims to improve our understanding of the role of environmental antibiotic pollution in these processes. Specifically, we have studied the effects of pollution from drug manufacturing on bacterial resistance to fluoroquinolones and international travel as a means of propagation of resistant bacteria.

The aims for the different papers were as follows:

Paper I — To understand the genetic alterations of an extensively multi-resistant bacterium, isolated from an extremely antibiotic-contaminated treatment plant that receives wastewater from drug production by linking the resistance phenotype to acquired resistance genes and mutations.

Paper II — To identify and characterise novel fluoroquinolone resistance plasmids, which are transferable to a model human pathogen through conjugation, from a severely antibiotic-polluted Indian lake.

Paper III — To describe the abundance of fluoroquinolone resistance mutations in environmental *Escherichia* communities and investigate the link between the abundances and the fluoroquinolone pollution.

Paper IV — To determine the abundance of fluoroquinolone resistance mutations in *Escherichia* communities in the human gut microbiome and investigate how it is affected by travel from Sweden to India.

Methodological Considerations

Here, the different methods chosen in the included papers will be described and discussed. The focus is to motivate the choice of method and less emphasis is put on details. For more exhaustive information, please refer to the papers themselves.

Sampling

The bacterium studied in paper I was isolated from a water sample collected from the equilibrium tank at the WWTP plant in Patancheru, India. The equilibrium tank receives the industrial effluent, delivered from approximately 90 generic drug manufacturers, and mixes it before it continues to the treatment process. This step is applied to avoid large fluctuations in the composition of the input, which could disturb the treatment process. The isolate was part of a study of the antibiotic resistance phenotypes of bacteria collected within different parts of the plant by Marathe et al.[54], in which it was the most multi-resistant out of all the screened isolates. The typing of the strain was performed first by 16S rRNA gene sequencing, which showed a 99.5% nucleotide (nt) identity to the type strain of *Ochrobactrum intermedium* LMG3301^T. Because 16S is not sufficient to distinguish between different species within the *Ochrobactrum* genus[55, 56], the gene for recombinase subunit A (*recA*) was also sequenced, showing a 97.7% nt identity to *O. intermedium* LMG3301^T. The isolate was, therefore, identified as an *O. intermedium* and archived into the Culture Collection of Gothenburg (CCUG) as *O. intermedium* CCUG 57381.

The sediment samples in paper II were collected from two lake with a reported history of excessive fluoroquinolone pollution and located near Patancheru: Asanikunta Tank and Kazipally Lake (Fig. 1a-b). The bacterial communities residing in the lakes would have needed to adapt to the antibiotic selection pressure, and could, therefore, harbour novel transferable resistance elements. To test this hypothesis, plasmid capture from the sediments was performed using *gfp*-tagged *E. coli* str. CV601, resistant to kanamycin and rifampicin. Samples were also collected from two other Indian lakes in the region, Himayat sagar and Osman sagar, both of which have been used previously as drinking water reservoirs. Two Swedish lakes situated near Gothenburg were added as additional references,

Härlanda tjärn and Axlemosse, none of which received any industrial or WWTP effluent.

Sediment samples were also analysed in paper III. The stream receiving the treated effluent from the WWTP in Patancheru was sampled both upstream (two sites) and downstream (three sites) of the discharge pipe. To represent sites with little or no fluoroquinolone, we also collected Swedish samples from a stream in Skövde and a small highland lake, Valbergs öga (Fig. 1c). The stream in Sweden was sampled upstream and downstream from a municipal WWTP that was not linked to drug production, and the lake had no input of sewage, faeces from farmed animals, or industrial wastewater. At each site, multiple samples were collected to reduce the effect of within-site variability, and the stream samples were pooled for each sample site before further analysis. To quantify fluoroquinolones in the sediments, they were analysed for ciprofloxacin, difloxacin, enoxacin, enrofloxacin, lomefloxacin, ofloxacin, pefloxacin, and norfloxacin using liquid chromatography coupled to an ion trap mass spectrometer and electro spray interface (LC-ESI-IT-MSMS), as described previously[45].

The stream sediments had already been analysed by Kristiansson et al. for the presence of genes associated with antibiotic resistance and HGT using metagenomic DNA sequencing[45]. The study revealed an increase in the abundance of a number of resistance genes and mobile genetic elements downstream from the Indian WWTP compared to upstream. However, the mobile *qnr* genes, liked to quinolone resistance, were more prevalent upstream than downstream, despite the lower concentration of fluoroquinolone detected in these samples. Therefore, we hypothesised that the bacterial communities could have adapted to the fluoroquinolone selection pressure by chromosomal mutations in the target genes.

In paper IV, a different environment was studied for the abundance of chromosomal quinolone resistance mutations, namely the human gut. To determine the abundances of these mutations in the gut microbiome and to test if they are affected by international travel, paired faecal samples were collected from twelve Swedish students. Samples were taken before and after they visited India to study or work as interns for periods of 28-106 days (average 64 days). Sweden and India differ markedly in terms of their relative fluoroquinolone consumption[22, 57] and frequency of fluoroquinolone resistance[8, 34]; Sweden ranks lower on both measures. Therefore, we hypothesised that the bacterial communities in the human gut could become partly colonised by Indian gut bacteria and, thereby, less susceptible to fluoroquinolones, even in the absence of antibiotic treatment. We also had the opportunity to analyse chromosomal resistance mutations in faecal samples collected in the region surrounding Patancheru, analysed previously by

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b)

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c)

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FIG 1 ***a)*** *The polluted Asinikunta Tank and* **b)** *Kazipally Lake near Hyderabad, India.* **c)** *The remote highland lake Valbergs Öga near Ale, Sweden.*

Rutgersson et al. for the presence of *qnr* genes[58]. Twelve participants, all living in villages where no fluoroquinolones were detected in either soil or well water[58], were included in this study. The collection of Swedish samples was approved by the regional ethical review board in Umeå, Sweden (2011-357-32M), and the Indian sampling received institutional ethical clearance (IEC, National Centre for Cell Science, Pune, India).

Phenotypic Resistance

Etest® strips were used according to the manufacturer's protocol to determine the antibiotic susceptibility of both the *O. intermedium* strains in paper I and the recipient strain and transconjugant strains in paper II. In paper I, the MIC of 47 antibiotics belonging to different classes – including aminoglycosides, β-lactams (carbapenems, cephalosporins, and penicillins), macrolides, fluoroquinolones, sulphonamides, and tetracyclines – was determined for both *O. intermedium* CCUG 57381 and the type strain *O. intermedium* LMG3301^T. In paper II, the recipient strain was grown in the presence of kanamycin, and the transconjugants in ciprofloxacin, carbenicillin, or sulfamethoxazole before the Etests. The tested antibiotics included ciprofloxacin, sulfamethoxazole, ampicillin, ceftazidime, streptomycin, tetracycline, and azithromycin. The bacterial communities of the sampled lakes were also tested for phenotypic resistance. Serial dilutions of sediment were grown on control plates with no antibiotics, and on plates with either ciprofloxacin or sulfamethoxazole. The numbers of colony forming units (CFU) were determined for each plate, and the results for the antibiotic plates were compared to their corresponding control plates.

DNA Extraction and Amplification

Whole-genome DNA was isolated from *O. intermedium* CCUG 57381 in paper I using a traditional method introduced by Marmur in 1961[59]. However, the method is laborious; therefore, in paper II, the QIAGEN DNeasy Blood and Tissue Kit was used for the DNA extraction from the recipient strain and transconjugant strains. Additionally, plasmid DNA was extracted from the transconjugants using the QIAGEN Plasmid Mini Kit with modifications of buffer volumes and incubation procedures. The plasmid DNA was used for restriction analysis, digested with enzymes PstI and Bst1107, and visualised on a gel. Furthermore, PCR primers targeting a sulphonamide resistance gene (*sul1*) and mobile quinolone resistances genes (*qnrABCDS* and *qnrVC*) were used to detect the presence of these genes in the transconjugants. In paper III, metagenomic DNA was isolated from the stream and lake sediment samples using the PowerSoil DNA Isolation Kit (MO BIO Laboratories, Inc.) with slight modifications to the attached protocol. The

extraction from the sediment samples had a low DNA yield and, hence, uniform whole-genome amplification was performed to increase the amount of isolated DNA, using the REPL-g Mini Kit (QIAGEN). The QIAamp DNA Stool Mini KIT (QIAGEN) was used for the stool samples in paper IV.

The PCR primers used in papers III and IV were designed with the aim of targeting the QRDRs of the genes *gyrA* and *parC* in *Escherichia* communities. To capture the largest possible proportion of the communities, all of the *Escherichia* sequences annotated as *gyrA* or *parC* in the Pathosystems Resource Integration Center (PATRIC)[60] were downloaded and aligned using MUSCLE[61]. Because *Escherichia* and *Shigella* genera are indistinguishable in the targeted region, all *Shigella* sequences annotated as *gyrA* or *parC* were also added to each alignment. In the primer design software Primer3Plus[62], for each gene, the region containing all known resistance mutations listed by Ruiz[19] was given as a target, and all non-conserved positions in the multiple sequence alignment were given as excluded regions. The top ten suggested primers were tested experimentally and the resulting amplicons separated on a gel. For each gene, the primer pair producing the largest amount of amplicon of the correct length was chosen.

Sequencing

For the first whole-genome sequencing study, described in paper I, we compared the Indian isolate, *O. intermedium* CCUG 57381, to the type strain, *O. intermedium* LMG3301^T, with the intention to link the acquired resistance phenotypes to resistance genes and mutations. Because the genome of the type strain had been previously sequenced, assembled and made publicly available, we decided to analyse the sequencing data by mapping all reads to the genome of the type strain, followed by a *de novo* assembly of unmapped reads. At the time (2009), massively parallel pyrosequencing[63](454 sequencing) generated, on average, 450 megabases (Mb) of data per sequencing run with reads of length 330 bp[64]. The relatively long 454 reads were deemed suitable for the project, especially for the *de novo* assembly of novel genetic material such as plasmids. This platform struggles with errors in regions with repetitive bases (homopolymers), but a sufficient sequencing coverage of the genome should still give a draft assembly of satisfactory quality. Therefore, the Roche GS FLX system was used with titanium chemistry and generated 90.6 Mb of data distributed over 249 120 reads, with an average read length of 364 bp. In paper II, the genomes of the recipient strain and selected transconjugant strains were sequenced with the aim of describing the genetic context of the acquired antibiotic resistance genes in the transconjugants. Even though the aim of the sequencing in paper II was similar to that of paper I, a different sequencing platform was chosen due to the technology advances during the four years that had passed

between the projects. Illumina sequencing had increased their sequence length from 100 bp to 250 bp, generating 600 gigabases (Gb) of data each run; therefore, the Illumina MiSeq system was deemed the most cost effective approach. Sequence quality deteriorated towards the end of the Illumina reads, hence, quality filtering and trimming of low quality reads is crucial. On average, 1.1 million paired 250 bp reads were generated for each sequenced strain.

For the amplicon sequencing in papers III and IV, 454 sequencing was used. In particular, because the longer read length enabled us to cover the entire QRDR of the genes of interest within a single read (amplicon lengths: 311 bp for *gyrA* and 287 bp for *parC*), allowing us to study the co-occurrences of multiple mutations in one amplicon. Furthermore, Illumina sequencing has difficulty to handle samples of low sequence variability, such as amplicon samples. To combat these restrictions, the amplicons are be diluted with a more complex library and those data are filtered out before data delivery[65]. Therefore, we used the Roche GS FLX system with titanium chemistry in both papers III and IV.

Data Analysis

The mapping of reads onto the genome of the type strain was performed using the BLAST-like alignment tool (BLAT)[66]. The tool is adapted to aligning short query sequences (reads) onto long database sequences (a reference genome), is capable of handling longer deletions in the sequenced bacterium, and has been previously utilised in the re-sequencing of the human genome using 454 sequencing[67]. Additionally, the reasonable amounts of data produced by the sequencing made BLAT a good choice of alignment tool. Reads not mapping to the genome of the type strain were assembled *de novo* using the 454 tool GS *De Novo* Assembler because it is optimised to handle 454 data with their associated types of sequencing errors. To identify acquired mobile resistance genes, the genome sequence of the type strain and all *de novo* assembled contiguous sequences (contigs) were matched against the Antibiotic Resistance Genes Database (ARDB)[68] using BLASTx[69] and all non-overlapping hits were assessed manually. Chromosomal non-synonymous mutations in the proteins GyrA, GyrB, ParC, and ParE (all linked to fluoroquinolone resistance), as well as in ribosomal protein S12 (associated with streptomycin resistance), were identified by matching all reads to reference sequences of each gene, using tBLASTn. The method was repeated to find mutations in the ribosomal rRNA genes 16S (linked to streptomycin resistance), and 23S (related to macrolide resistance), but by instead using nBLAST. To identify genes involved in conjugation by type IV secretion systems (T4SS), all *de novo* assembled contigs were analysed using CONJscan[70], insertion sequences (IS:s) were detected using IS-finder with BLASTx (http://www-is.biotoul.fr), and

insertion sequence common regions (ISCRs) were screened for by using BLASTn against all sequences listed by Toleman et al. [71].

In paper II, the Illumina reads from each whole-genome sequenced strain were trimmed for adaptor sequences, quality filtered, and trimmed using Trim Galore! (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) with a quality threshold of Phred score 20. In an evaluation of several *de novo* assemblers used on bacterial whole-genome Illumina sequencing data, SPAades[72] was considered the best tool for the assembly of MiSeq data[73]. It was also the assembler that managed to produce the most contigs in which entire protein coding sequences were contained, which is a valuable property when searching for antibiotic resistance genes. Therefore, all reads passing the filtering, both paired reads and reads that lost their paired read in the filtering, were aligned appropriately using SPAdes 2.5.1 with mismatch correction. Antibiotic resistance genes were identified by mapping the resulting contigs against the Resqu database (version 1.1, http://www.geneway.net/resdb) using HMMer (version 3.1b1)[74] with all six possible reading frames. The Resqu database is composed of 3 018 non-redundant protein sequences corresponding to 325 resistance gene families, all of which have been manually retrieved from the literature. Furthermore, the draft assemblies were annotated for genes involved in conjugation using the HMM profiles described by Smillie et al.[17] and Guglielmini et al.[75]. To find non-synonymous mutations in *gyrA*, *gyrB*, *parC*, *parE*, and ribosomal proteins S12, L4, and L22, the draft assemblies were mapped to reference protein sequences from *E. coli* K-12 MG1655, using tBLASTn. Mutations in the ribosomal RNA subunits 16S and 23S were detected by mapping the quality filtered reads for each transconjugant onto reference sequences of both genes from *E. coli* K-12 MG1655, using Bowtie (version 1.0.0)[76]. The results were screened for SNPs using SAMtools (version 0.1.19)[77]. The results of the transconjugant strains were compared to the recipient strain to determine acquired genes and mutations. Because we were also interested in the genetic context of the acquired mobile resistance genes, all contigs from the transconjugant strains carrying resistance genes were submitted for automatic annotation using RAST[78]. To find if any transconjugants carried similar plasmids, the resistance contigs were also mapped against each other using Mugsy (version 1.2.3)[79] with one input file per transconjugant. The multiple alignment was visualised using Gmaj (release 2008-Jun-30)[80].

To determine the abundance of resistance mutations in *Escherichia gyrA* and *parC* in the sampled external environments and human guts, the 454 sequencing data were first aligned to their respective reference sequences (from *E. coli* K-12 MG1655), using the GS Amplicon Variant Analyzer (AVA) from 454. This tool is optimised to detect amplicon variants from 454 amplicon sequencing data, and it was, therefore, well-suited for both papers III and IV. However, because we were

interested only in non-synonymous mutations, the resulting alignment required further analysis. First, one multiple alignment file was exported from AVA for each sample. Because complex samples of diverse bacterial communities were sampled, there was a risk of capturing non-*Escherichia* sequences and interpreting inter-genus variability as the result of fluoroquinolone pollution (paper III) or of travels to India (paper IV). Therefore, nBLAST was used to map all raw reads against all sequences annotated as *gyrA* or *parC*, respectively, in the Comprehensive Microbial Resource (CMR)[81] – a database containing collective annotations of bacterial genomes – and only sequences with the best hit against *Escherichia* or *Shigella* were retained in the subsequent analysis. Next, to minimise the impact of homopolymer errors on frame shifts, all gapped positions in the reference sequence were removed from the multiple alignment and any remaining gaps in the amplicon sequences were substituted with the reference base. Finally, the resulting ungapped multiple sequence alignment was translated using the EMBOSS tool transeq[82]. The abundance of amino acid substitutions were recorded as the percentage of the total number of analysed reads in the sample.

Results and Discussion

The results of the included papers will be summarised and discussed in a rather broad context. The results of papers I and II, both utilising whole-genome sequencing, will be considered first, then papers III and IV, which employed amplicon sequencing, will be discussed. For detailed information on all results and a more in-depth discussion of them, please refer to the included papers.

Whole-Genome Sequencing

In papers I and II, whole-genome sequencing was used to study the genetic mechanism behind acquired antibiotic resistance. The screened antibiotic mechanisms included both mobile resistance genes and chromosomal mutations in target genes. To evaluate the potential for mobility of the detected resistance genes, all studied genomes were also screened for genes involved in plasmid conjugation and other genetic elements involved in HGT.

Multi-resistant bacterium isolated from an industrial WWTP

Ochrobactrum intermedium has been shown to have a highly variable genome, and it carries between zero and three three plasmids[83]. The genome of the type strain (*O. intermedium* LMG 3301^T), used as a reference in paper I, consists of two chromosomes (2 604 Mb and 1 920 Mb, respectively) and two plasmids (141 Mb and 60 Mb, respectively). The mapping of sequence reads from the Indian strain (*O. intermedium* CCUG 57381) onto the reference genome covered 99% of the chromosomes and the large plasmid. The practically non-existent coverage of the small plasmid indicated that it was not present in the Indian strain. The 1% of the reference genome left uncovered in the mapping consisted mainly of larger regions, primarily in the two chromosomes, which suggested large, putative deletions in the Indian strain. The mapping results enabled the search for resistance mutations in chromosomal genes known to be linked to resistance. The reads not matching the reference genome (18% of all reads) were *de novo* assembled to study novel genetic material, especially acquired resistance genes.

The phenotypic screening of the Indian strain and the reference strain showed that both strains were highly multi-resistant. Indeed, the species has been shown to be intrinsically highly resistant to a large number of antibiotic classes including the

aminoglycosides (amikacin, MIC_{50}=64 mg/l), penicillins (amoxicillin/clavulanic acid, MIC_{50}>256 mg/l), macrolides (azithromycin, MIC_{50}>250 mg/l), cephalosporins (cefepime, MIC_{50}>256 mg/l), and amphenicols (chloramphenicol, MIC_{50}=32 mg/l)[84]. However, the Indian strain had acquired additional resistance towards fluoroquinolones, tetracyclines, and sulphonamides. Table 1 lists all tested antibiotics where there was at least a 3-fold difference in the MIC of the Indian strain and the reference strain, as well as the related acquired genotypes.

Quinolone resistance is caused by chromosomal mutations in the target genes, especially *gyrA* and *parC*, or through the acquisition of mobile plasmid-mediated resistance genes, such as *qnr*, *qepA*, and *aac(6')-lb-cr*. The mapping revealed non-synonymous mutations in *gyrA*, *parC*, and *parE* in the Indian strain when compared to the reference strain. There were four mutations in *gyrA*, causing the amino acid substitutions: A83V, S84P, S206A, and A865G (using *E. coli* numbering); three in *parC*, resulting in the substitutions A80V, E205D and D439E; and one in *parE*, giving R135H. No non-synonymous mutations were detected in *gyrB*. Codons 83 and 84 in *gyrA* and codon 80 in parC have previously been linked to quinolone

TABLE 1 *Antibiotic resistance phenotype and genotype of the screened O. intermedium strains CCUG 57381 and LMG 3301^T*

	MIC (mg/L)		
Antibiotics	**Indian strain**	**Reference strain**	**Acquired genotype**
Aminoglycosides			
Streptomycin	32	8	-
Macrolides			
Azithromycin	32	2	
Clarithromycin	>256	8	-
Erythromycin	>256	24	
Quinolones			
Nalidixic acid (1st gen.)	>256	16	
Ciprofloxacin (2nd gen.)	>32	0.25	Amino acid
Ofloxacin (2nd gen.)	>32	0.5	substitutions in the
Levofloxacin (3rd gen.)	>32	0.25	target proteins of
Gatifloxacin (4th gen.)	>32	0.5	quinolones
Moxifloxacin (4th gen.)	>32	2	
Sulphonamides and DHFR inhibitors			
Sulfamethoxazole	>1024	2	*sul1*
Trimethoprim/Sulfamethoxazole	>32	0.064	
Tetracyclines			
Doxycycline	24	1.5	*tet(G)*
Tetracycline	24	1.5	

resistance in *E. coli*[24]. Indeed, codon 83 is the most frequently detected mutated codon in *gyrA* in fluoroquinolone-resistant clinical *E. coli* isolates, and codon 80 is the equivalent in *parC*. A multiple sequence alignment of the homologous proteins GyrA and ParC from *O. intermedium* LMG3301^T and *E. coli* K-12 MG1655 not only confirms that position 83 in GyrA corresponds to position 80 in ParC[24] but also shows that the QRDRs are highly similar (Fig. 2). Therefore, the non-synonymous mutations in *gyrA* and *parC* offer a plausible genotype causing the acquired fluoroquinolone resistance.

Tetracyclines are broad-spectrum antibiotics that target the protein synthesis in a bacterium by binding to the ribosomal 30S subunit[85]. Resistance is mainly caused by the acquisition of mobile resistance genes, including pumps for active efflux of the drug, ribosomal protection proteins, and enzymatic inactivation of the drug[86]. The *de novo* assembly of novel genetic material acquired by the Indian strain, resulted in a contig containing the tetracycline efflux pump gene *tet(G)* and its repressor gene *tetR*. The gene is known to confer resistance to tetracycline and doxycycline[87], which is in line with our data. Furthermore, efflux pumps rarely protect the bacterium against glycylcyclines such as tigecycline, and there was no difference between the Indian strain and the reference strain in the MIC of the glycylcycline tigecycline. Hence, the acquisition of the *tet(G)* and *tetR* genes by the Indian strain is likely the cause of the observed tetracycline resistance profile. There was also a contig containing the sulphonamide resistance gene *sul1*. Sulphonamides target folate synthesis by competitive inhibition of the enzyme dihydropteroate synthetase and are bacteriostatic[88]. The *sul1* gene encodes a variant of the enzyme that is unaffected by the sulphonamides and confers a high level of resistance, as observed in the Indian strain. The reference strain was intrinsically resistant towards trimethoprim but sensitive to the sulphonamide sulfamethoxazole and the combination of the two antibiotics. Therefore, the high level of resistance to the combination trimethoprim/sulfamethoxazole in the Indian strain is likely also caused by the acquisition of the *sul1* gene.

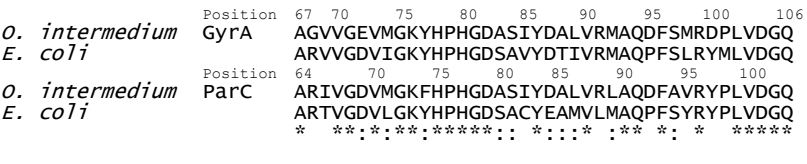

FIG 2 *Multiple sequence alignment of the quinolone-resistance determining regions (QRDRs) of GyrA and ParC in O. intermedium LMG 3301^T and E. coli K-12 MG1655^T reveals a high sequence similarity. Positions are given using E. coli numbering, positions that are completely conserved are marked below with an asterisk (*), and the colon (:) indicates conservation within a group of amino acids with strongly similar properties.*

There was also an observed difference in the measured MICs of all tested macrolides and the aminoglycoside streptomycin, both of which target protein synthesis. For macrolides, the most common resistance mechanism involves the methylation of the 23S rRNA[89]. Additionally, there are chromosomal mutations in the 23S rRNA gene and ribosomal protein L4, as well as mobile genes responsible for the efflux and enzymatic inactivation of the drug. The Indian strain had not acquired any mobile macrolide resistance genes, nor were there any mutations in the 23S rRNA gene in positions previously linked to resistance. The broad-spectrum aminoglycoside streptomycin exercises its bactericidal effect by binding to the 16S rRNA. Streptomycin resistance is associated with chromosomal mutations in the 16S rRNA gene and the ribosomal protein S12, as well as the acquisition of mobile aminoglycoside-modifying enzymes[90]. The Indian isolate had no amino acid substitutions in the ribosomal protein S12 and had not acquired any streptomycin resistance genes. In addition, all alterations in the 16S rRNA gene were found in positions far from regions associated with streptomycin resistance. However, both the MICs of the macrolides and streptomycin are within the range previously reported from *O. intermedium*[84] and could, therefore, be caused by differential expression of pre-existing genes or mutations in general efflux pumps. There is also the possibility that the Indian strain had acquired a novel resistance mechanism for the antibiotics in question.

In the novel genetic material obtained by the Indian strain, there were also genes involved in horizontal gene transfer. For a plasmid to be conjugative, it must carry MOB genes and a MPF complex[17]. The Indian strain had acquired a MOB_P relaxase and genes belonging to MPF_I (*traIMNY*), suggesting that it has gained a plasmid that was not present in the reference strain. Furthermore, two additional insertion sequences (ISs) were detected in the Indian strain, ISApr9 and ISRle4. There was also a high sequence similarity between a 4.7 kb region in the contig containing *tet(G)* and Salmonella genomic island (SGI-J)[91]. It is likely that some of these mobile genetic elements are responsible for the transfer of the acquired resistance genes.

To recapitulate, the Indian strain, *O. intermedium* CCUG 57381, had acquired three distinct resistance phenotypes when compared to other isolates of the same species: quinolone, sulphonamide, and tetracycline resistance[84]. It is likely that these traits have given the strain a selective advantage in the extreme environment from which it was isolated. Our results show the genetic basis in which an environmental bacterium can acquire resistance and that industrial WWTPs can form hot-spots where the survival and replication of antibiotic-resistant bacteria are promoted. Furthermore, the studied WWTP also receives human sewage as part of the microbiological treatment process, increasing the risk of HGT of mobile resistance from environmental bacteria to human pathogens. This transfer could

also occur in the human gut, which *O. intermedium* is known to inhabit[92]. To avoid these transfer events, it is important to limit antibiotic exposure in environmental bacterial communities.

Resistance plasmids from an antibiotic polluted lake

The cultures from the lake samples showed an increased abundance of resistant bacteria from Kazipally Lake and Asanikunta Tank compared to the Indian and Swedish reference lakes. Both lakes have a history of fluoroquinolone pollution, and showed a significantly higher frequency of bacteria resistant to either ciprofloxacin or sulfamethoxazole compared to the Indian and Swedish reference lakes. This result indicate that the antibiotic pollution in both contaminated lakes has selected for resistant strains.

All the transfer experiments between the sampled bacterial communities from Kazipally Lake and the *gfp*-tagged *E. coli* recipient strain resulted in transconjugants with acquired ciprofloxacin, sulfamethoxazole, or carbenicillin resistance. No transconjugants with acquired resistance were obtained from any of the reference lakes. For Asanikunta Tank, the toxicity of the samples was too severe for the recipient strain and, therefore, there was no growth, even on the reference plates. From the Kazipally Lake samples, 30 transconjugant colonies from the ciprofloxacin plates (titled ci-tc 2 and ci-tc 5-33), 16 from the sulfamethoxazole plates (sx-tc 1-16), and 8 from the carbenicillin plates (ca-tc 1-8) were selected for further analysis. The selected colonies were screened for additional acquired antibiotic resistance phenotypes. None of the sulfamethoxazole transconjugants was more resistant to any of the tested antibiotics except for sulfamethoxazole, when compared to the recipient strain. Therefore, none of the sulfamethoxazole transconjugants were further studied. All but one of the carbenicillin transconjugants (ca-tc 2) showed a similar resistance pattern and a similar restriction fragment pattern. Transconjugant ca-tc 2 had also acquired resistance to streptomycin and had a lower level of resistance to ceftazidime compared to the other carbenicillin transconjugants. Therefore, both ca-tc 2 and ca-tc 6 were selected for further analysis. From the ciprofloxacin transconjugants, the isolated plasmids showed seven distinct restriction fragment patterns (A-G). For restriction fragment patterns D and G, there was only one transconjugant representing: ci-tc 23 and ci-tc 33, respectively. For patterns B, C, and F, all the represented transconjugants showed similar resistance phenotypes and only one transconjugant was selected for each restriction pattern: ci-tc 9, ci-tc 13, and ci-tc 30, respectively. Despite having similar restriction fragment patterns and resistance phenotypes, both ci-tc 2 and ci-tc 24 (restriction fragment pattern A) and ci-tc 29 and ci-tc 31 (E) were picked for further investigation. In all, eleven transconjugants were selected for whole-genome sequencing, together with the recipient strain.

The twelve draft assemblies of the sequenced genomes were screened for antibiotic resistance genes, and, expectedly, all transconjugants had acquired resistance genes. In table 2, all the MICs of the recipient strain and the MICs of the transconjugants that were at least 3-fold higher than that of the recipient strain are given. There were six transconjugants with an increased resistance to the β-lactams ampicillin and ceftazidime. Most β-lactams inhibit cell wall synthesis by binding to penicillin-binding proteins (PBPs), and they can be further divided into the classes penicillins, cephalosporins, monobactams, and carbapenems. Resistance mechanisms include enzymatic inactivation of the drug by β-lactamases and alteration of the PBPs[93]. All six of the transconjugants with reduced susceptibility carried genes encoding β-lactamases of groups OXA, SHV, and VEB. The bla_{OXA-10} belongs to a family of β-lactamases that are characterised by their ability to hydrolyse the penicillins cloxacillin or oxacillin rapidly[94]. The acquired bla_{SHV-12} gene belongs to a functional class of extended spectrum β-lactamases (ESBLs) that readily hydrolyses penicillins, in addition to cephalosporins of early generations and some of later generations. However, these enzymes are sensitive to the β-lactamase inhibitors clavulanic acid and tazobactam. The bla_{VEB-9} β-lactamase is also an ESBL and sensitive to clavulanic acid inhibition. The acquisition of the β-lactamase genes by transconjugants ci-tc 29, ci-tc 31, ci-tc 33, ca-tc 6 (bla_{VEB-9}), ci-tc 30, and ca-tc 2 (bla_{OXA-10} and bla_{SHV}) explains their increased resistance to the tested β-lactam antibiotics.

All sequenced transconjugants carried mobile quinolone resistance genes: either variants of *qnrS* or *qnrVC1*. Notably, this is the first time *qnrVC1* – previously detected only in chromosomes – is described on a conjugative plasmid. The *qnr* genes are known to confer moderate levels of resistance to fluoroquinolones[95], which is in line with our finding that the largest ciprofloxacin MIC was 1.5 mg/L. It should, however, be stressed that the clinical breakpoint for resistance to ciprofloxacin in Enterobacteriaceae is 1 mg/l according to EUCAST[31]. No non-synonymous mutations were detected in the target genes *gyrA*, *gyrB*, *parC* or *parE* in either of the transconjugants. The accusation of qnr genes by all transconjugants is, hence, a plausible cause of their fluoroquinolone resistance. Furthermore, all but three transconjugants had gained resistance to sulfamethoxazole. This phenotype could be linked to the acquisition of the sulphonamide insensitive dihydropteroate synthetases *sul1* (ci-tc 30, ci-tc 33, and ca-tc 2), *sul2* (ci-tc 2 and ci-tc 24), or both genes (ci-tc 29, ci-tc 31, and ca-tc 6). Two transconjugants had acquired streptomycin resistance (ci-tc 30 and ca-tc 2) that could be linked to the gain of the aminoglycoside resistance gene *ant(3'')-Ia*. The gene encodes an enzyme that adenylylates and thereby inactivates streptomycin and spectinomycin[96]. Because there were no chromosomal alterations in the genes linked to streptomycin resistance, the acquisition of *ant(3'')-Ia* is likely the cause of the resistance. A single

TABLE 2 *Antibiotic resistance phenotype and genotype of the screened E. coli CV601 recipient strain and transconjugant strains selected for sequencing*

	MIC (mg/l)												
Antibiotic	Recipient	ci-tc 2	ci-tc 9	ci-tc 13	ci-tc 23	ci-tc 24	ci-tc 29	ci-tc 30	ci-tc 31	ci-tc 33	ca-tc 2	ca-tc 6	**Acquired genotype**
Ampicillin	8						>256		>256	>256		>256	*bla*$_{VEB}$
								>256			>256		*bla*$_{OXA-10}$, *bla*$_{SHV-12}$
Azithromycin	3	12				24							-
Ceftazidime	0.38						>256		>256	48		>256	*bla*$_{VEB}$
								24			16		*bla*$_{OXA-10}$, *bla*$_{SHV-12}$
Ciprofloxacin	0.023	0.75	0.75	0.75	1	1.5	1.5	0.75	1	1	0.5	0.75	*qnrS1* *qnrS2* *qnrVC1*
Streptomycin	3							64			64		*ant(3")-Ia*
Sulfamethoxazole	16	>1024				>1024		>1024		>1024	>1024		*sul1* *sul2*
							>1024		>1024			>1024	*sul1*, *sul2*
Tetracycline	1.5									12			*tet(C)*

transconjugant (ci-tc 33) was less susceptible to tetracycline compared to the recipient strain. The resistance was likely caused by the acquired *tet(C)* gene, which is an efflux pump know to confer resistance to tetracycline[85]. Finally, there were two transconjugants that had gained resistance to the macrolide azithromycin, but no macrolide resistance genes or mutations were detected in either isolate.

When comparing all contigs containing acquired resistance genes, the high sequence similarity among contigs originating from different transconjugants indicated that some were carrying identical or highly similar plasmids. The carbenicillin transconjugants differed from each other, but there was a high sequence similarity between the resistance contigs in ca-tc 2 and ci-tc 30 and between ca-tc 6 and ci-tc 31. The similarities between these pairs were also evident for the phenotypic resistance pattern and acquired resistance genes. Moreover, there was a high sequence similarity between ci-tc2 and ci-tc 24, which also showed comparable resistance phenotypes and restriction fragment patterns, and had acquired the same antibiotic resistance genes. However, even if the same could be said about ci-tc 29 and ci-tc 31, there were slight differences in the sequences. The sequence alignment suggested that ci-tc 31 had either acquired more genetic material or that a larger proportion of the plasmid had been assembled. Finally,

transconjugants ci-tc 9, ci-tc 13, and ci-tc 23 shared resistance phenotypes and a high sequence similarity but had different restriction fragment patterns. This could be due to slight differences in the sequence causing alterations in the restriction recognition site.

The detection of genes involved in conjugation revealed that all draft assemblies contained genes encoding relaxases and MPF proteins. According to the classification scheme proposed by Smillie et al., transconjugants ci-tc 9, ci-tc 13, and ci-tc 23 had acquired a MOB_F relaxase and a MPF_T complex, and all other transconjugants had gained a MOB_H relaxase and a MPF_F complex. The contigs containing conjugation genes were further investigated by mapping to NCBI's GenBank using BLASTn. The results indicated that the two groups of plasmids likely belonged to the incompatibility groups IncN and IncA/C, respectively. The result was further supported by the presence of corresponding plasmid replication gene, *repA,* in all draft assemblies. All transconjugants carrying MOB_F and MPF_T encoded a protein with 100% amino acid (aa) identity to RepA of the IncN reference plasmid R46. The remaining transconjugants, however, encoded proteins with a 98-99% aa identity to RepA of the IncA/C reference plasmid pRA1, and had an additional 78 amino acids at the C-terminal end. Therefore, the assignment of these plasmids to incompatibility group IncA/C needs to be further evaluated.

The results suggest that there are at least seven different plasmids isolated from Kazipally Lake, all conferring resistance to ciprofloxacin and some also to ampicillin, azithromycin, ceftazidime, streptomycin, sulfamethoxazole, or tetracycline. To determine the exact number of plasmids isolated, additional sequencing efforts to close the gaps will be required. Here we show that environments polluted with antibiotics can act as breeding grounds for the selection and dissemination of mobile antibiotic resistance determinants. The plasmids isolated in paper II are conjugative and transferable to a human pathogen model organism, showing that there is a risk for the transfer of resistance determinants from pollution hot-spots to the clinic.

Predicting phenotype from genotype

The results in papers I and II both indicate that, given a reference genome with a known corresponding resistance phenotype, it is possible to predict acquired resistance using massively parallel whole-genome sequencing. However, the presence of a gene that is highly similar to an antibiotic resistance gene in a genome does not necessarily prove that the bacterium is resistant to the corresponding antibiotic. Small changes can easily change the function of the encoded protein. A single substitution can, for example, transform the β-lactamase $bla_{SHV\text{-}1}$ into an ESBL and increase the MIC from 0.125 to 16 mg/L for the third generation

cephalosporin cefotaxime in *E. coli*. It is also possible that the presumed resistance gene is insufficiently expressed or not expressed at all. Furthermore, although a gene protects certain bacteria from antibiotics, it may not perform the same function in other bacteria within different genomic contexts[97]. Despite these possible confounding factors, the results of papers I and II indicate that screening for antibiotic resistance using sequencing is a feasible method. This is in accordance with a study of 197 strains isolated from pig manure that were sequenced and screened for antibiotic resistance[98]. Out of the 3 051 determined phenotypes, 99.7% could be predicted by the detection of antibiotic resistance genes. Six of the seven faulty predictions were of spectinomycin (an aminoglycoside) resistance in *E. coli*. On the other hand, we struggled to explain acquired macrolide resistance in both papers I and II. As mentioned, one of the resistance mechanisms against macrolides involves the methylation of target nucleotides in the ribosomal 23S subunit. There are specific enzymes know to perform this modification, *erm* genes, but none of these were detected in either paper I or paper II. However, because the methylation state of the 23S rRNA cannot be inferred from the DNA sequence data, it is not possible to tell if the rRNA had been methylated by a different enzyme. This enzyme could be a novel macrolide resistance gene or a pre-existing gene with, for example, an altered expression or mutations that change its normal function.

Amplicon Sequencing of Quinolone Target Genes *gyrA* and *parC*

The method used in papers III and IV were identical, but the studied environments – sediment from aquatic environments under different fluoroquinolone selective pressure and the human gut – are vastly different. Here, the two datasets have been combined and discussed together. The results showed a surprisingly high abundance of the resistance mutations most commonly detected in clinical isolates.

Abundance of resistance mutations

The filtered amplicons were compared to reference sequences from *E. coli* K-12 MG1655 and analysed for mutations causing amino acid substitutions. The substitution abundance in a sample was measured as the number of amplicons carrying a non-synonymous mutation per total number of analysed amplicons in that sample. There were three amino acid substitutions considerably more abundant in all samples than all other substitutions: S83L and D87N in GyrA and S80I in ParC, all of which have been previously linked to quinolone resistance. The average abundance of the substitutions S83L and D87N was 63% and 23%, respectively. The S83L substitution is the most frequently detected substitution in fluoroquinolone-resistant clinical *E. coli* isolates followed by the D87N substitution[24]. The occurrence of both substitutions in the same amplicon had an

average abundance of 22% because almost all of the amplicons containing D87N also carried S83L. In ParC, the most frequently detected substitution in clinical *E. coli* isolates resistant to fluoroquinolones is S80I[24], which was detected at an abundance of 20%, on average. In Fig. 3, the data for the substitutions mentioned above are given for each sample, grouped according to the origin of the

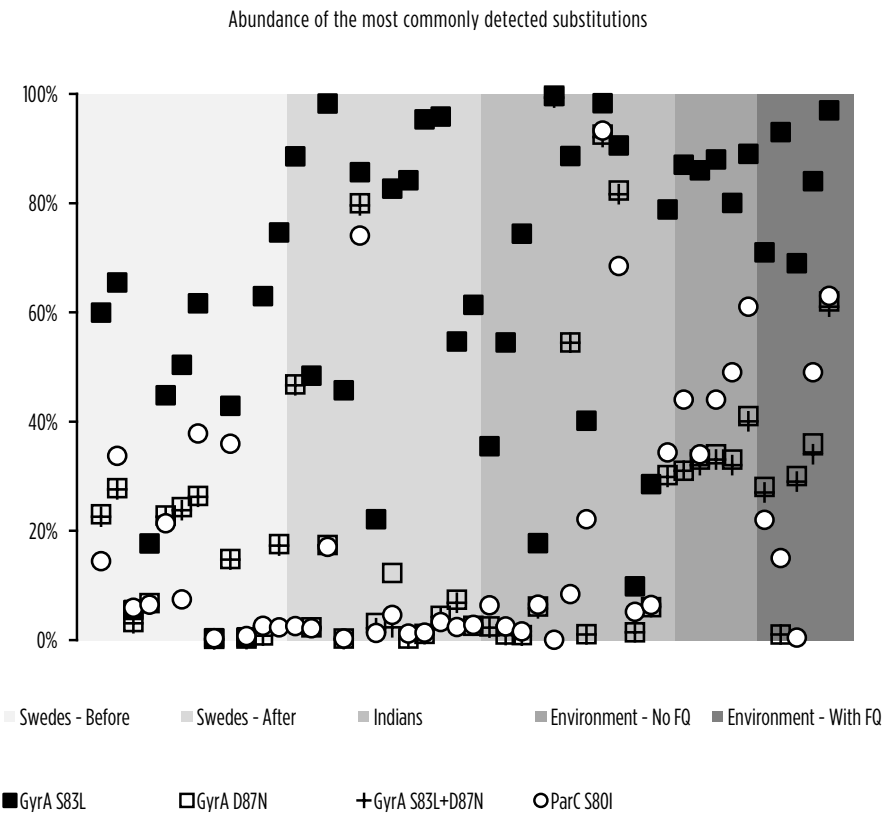

FIG 3 *Abundance of the most commonly detected amino acid substitutions in the quinolone-resistance determining region of GyrA (S83L, black squares; D87N, open squares; and the combination thereof, plus sign) and ParC (S80I, grey circles). The vertical background stripes represent the different sample groups. From the left: Swedish guts sampled before travel to India, Swedish guts sampled after return, Indian guts, sediment samples collected from environment with no detected fluoroquinolones, and sediment samples collected in fluoroquinolone polluted environments (71.7-1 030 µg fluoroquinolones/g organic matter).*

sample. The data clearly show that these mutations are naturally plentiful in *Escherichia* communities, even where there is no fluoroquinolone present. This result suggests that there could be additional fitness benefits linked to these substitutions, other than providing resistance to quinolones. The data also show great variability, even between samples of similar origin. The least variability appears within the samples taken from external environments with no detected fluoroquinolone pollution (second section from the right in Fig. 3). However, three of the five samples in this group were collected from the same small lake, and these samples were expected to be highly similar. The clinical break point for ciprofloxacin resistance in Enterobacteriaceae is an MIC of 1 mg/L, according to EUCAST[31]. It has been shown that, in laboratory settings, a combination of the substitutions S83L in GyrA and S80I in ParC confers a ciprofloxacin MIC of 1 mg/L in *E. coli*[99]. If the D87N substitution in GyrA is also acquired, the MIC is 32 mg/L. Amplicon sequencing of multiple genes from bacterial communities does not provide information about which mutations in different genes occur in the same bacterium. However, the proportion of *Escherichia* strains with the potential for clinical resistance could, on average, be up to 46% in the Swedish environmental samples and up to 14% in the Swedish guts sampled before the visit to India. The frequency of invasive, fluoroquinolone-resistant *E. coli* infections in Sweden was 11.2% in 2012[8]. It is likely that the distribution of genotypes (and corresponding phenotypes) differ among pathogenic *E. coli* and environmental populations for two reasons: first, *E. coli* is known to have a plastic genome[100], and second, the populations inhabit vastly difference niches. As far as we know, this is the first time someone has reported the abundance of fluoroquinolone resistance mutations in the external environment and the human gut, using large scale techniques that do not rely on the culturability of the bacteria.

The effect of fluoroquinolone pollution

The chemical analysis of the stream and lake sediment showed that the sampled environments represented a wide range of fluoroquinolone pollution. As shown previously, the sampled area downstream of the Indian WWTP was severely polluted (71.7-1 030 µg total fluoroquinolones/g organic matter), and the Indian upstream samples contained a moderate level of pollution (25.9-16.6 µg/g organic matter)[45]. The greatest amount was detected in the downstream sample collected closest to the discharge pipe of the Indian WWTP, with decreasing concentrations the further downstream the samples was taken. The upstream sample collected closest to the pipe was more contaminated than that collected further upstream. None of the Swedish samples contained any of the eight tested fluoroquinolones, but it is fair to assume that the samples collected from the stream receiving municipal WWTP effluent is subjected to a higher anthropogenic impact than the sampled lake (Fig. 1c).

Despite the large differences in detected fluoroquinolones, there was no correlation to the abundance of resistance mutations. The fluoroquinolone-polluted samples in figure 3 (darkest background area to the right) are arranged in increasing contamination order, so that the highest concentration of drugs was detected in the sample to the far right. This most polluted sample has, indeed, the highest abundance of S83L and D87N, alone and together, in GyrA, as well as S80I in ParC out of all the environmental samples. Therefore, one could argue that this was the only sampled site with a sufficiently high fluoroquinolone level to create a selective advantage for these resistance mutations. Even though 0.1% of all organic matter in a sample being fluoroquinolones sounds alarmingly high, the detected fluoroquinolones are not all necessarily bioavailable. Ciprofloxacin has, indeed, been shown to sorb to soil particles and, thereby, become less toxic for microorganisms[101]. The toxicity was not entirely lost and, interestingly, it did not correlate with the concentration of ciprofloxacin added to the soils (0.2-20 μg/g organic matter). The reference soil, however, had significantly higher growth, which implies that ciprofloxacin concentrations of ≥0.2 μg/g organic matter are sufficient to affect the microbiome. Despite this report, our data suggest that there is no selection for fluoroquinolone resistance mutations in the QRDR of *gyrA* or *parC* in *Escherichia* communities, comparing polluted to non-polluted samples. Either way, the results do not indicate that the abundances of chromosomal resistance mutations in the QRDR of either *gyrA* or *parC* are suitable biomarkers for fluoroquinolone pollution.

The effect of traveling

There was a significant increase in the abundance of the S83L substitution in the Swedish samples collected after the visit to India compared to the corresponding sampling before the visit ($p<0.05$). The acquisition of this single alteration is not sufficient for clinically relevant resistance[24], but it would bring the bacterium one mutation closer to high-level fluoroquinolone resistance. Certain infections of E. coli are thought to originate from the patient's own microbiome, and, therefore, carrying resistant bacteria in the gut could increase the risk of contracting a resistant infection. There was no significant difference in any of the other commonly detected substitutions. There was, however, a significant decrease in the number of observed genotypes of both *gyrA* and *parC* after the visit to India ($p<0.01$), measured as the number of unique (translated) amplicon sequences more abundant than 0.5% of the total number of amplicons analysed in a sample. This finding suggests that the diversity within the studied bacterial population was reduced during travel. None of the participants in the study were treated with antibiotics during their visit, and it is, therefore, unlikely that the original microbiota mutated and adapted as a consequence of increased antibiotic selection pressure. It is more likely that the microbiota was partially replaced by Indian

strains that are more adapted to the Indian microbiome, food, and environment, and that may have been shaped by the greater use of fluoroquinolones in Indian society over the years. All but one participant reported suffering from traveller's diarrhoea during their stay. This event most likely facilitated an exchange or partial replacement of the gut flora, and it is likely to have contributed to the differences we observed, both in the S83L substitution and the overall diversity. Our results show that not only are antibiotic resistance genes disseminated over the world by international travel, but the bacteria that carry chromosomal resistance mutations are as well.

Conclusions

This thesis has contributed to our understanding of how complex microbial communities adapt to antibiotic contamination of the environment. The strain *O. intermedium* CCUG 57381, sequenced in paper I, had acquired both chromosomal mutations and mobile resistance genes that are likely to contribute to its propagation in the highly toxic environment inside the WWTP in Patancheru. The genetic contexts in which some of the acquired resistance genes were found (e.g., SGI) are also observed in clinical isolates of other species, suggesting that there has been a flow of genetic material between the bacteria in the environment and pathogens. In paper II, the bacteria residing in Indian lakes with a history of severe antibiotic pollution had also adapted to the extreme environment. Comparisons to less polluted lakes using culturing showed that a larger fraction of the community could survive all tested antibiotics.

Places where the concentrations of antibiotics are sufficiently high can become hot-spots in which resistant environmental bacteria have a selective advantage. The polluted Indian lakes are clearly examples of such hot-spots. The Indian WWTP does not only have a very high concentration of antibiotics, it also receives human sewage as a part of the treatment process. The combination of selective concentrations of antibiotics and a great abundance of human pathogens, or other bacteria capable of colonising the human body, increases the risk for the transfer of undiscovered resistance factors from the environmental resistome into pathogenic bacteria. This transfer of resistance could also occur within the human gut from bacteria that can procreate both in the external environment and in or on the human body. The species *O. intermedium* is an example of such a bacterium because it has been found in the human microbiome[92]. The results of papers I and II highlight the risks to human health associated with antibiotic contamination of the external environment.

In papers III and IV, resistance mutations in *Escherichia* communities were studied. The high abundance of amino acid substitutions previously known to be associated with quinolone resistance and the low abundance of other substitutions was intriguing. The studied samples originated from environments with a wide range of fluoroquinolone-selective pressures: from severely polluted sediments to the guts

of volunteers that had not been treated with antibiotics for at least six months and all the way to an isolated, pristine highland lake in Sweden. Still, substitutions in codon 83 and 87 in *gyrA* and codon 80 in *parC* were detected in all samples. This finding suggests that these substitutions are not associated with any fitness cost for the bacterium or that there may even be a fitness gain in the studied environments. Indeed, this property has been shown in a laboratory setting, but our results indicate that it also holds for more complex environments. If so, it could provide an explanation for why fluoroquinolone resistance can emerge quickly, even during the treatment of an infection[2424].

All included papers have made use of massively parallel sequencing and bioinformatic analyses. The technological developments over the last decades in the area of sequencing have revolutionised how bacterial genetics are studied. Whole-genome sequencing allows researchers to study the genetic context of genes rather than just the single genes. Furthermore, metagenomics has enabled the study of entire bacterial communities, including bacteria that are difficult to culture using available laboratory methods. With each technological update, more data are generated for lower costs. This increase in data volume, however, intensifies the need for tools that can quickly and accurately analyse data. It also requires that the copious amounts of results are interpreted in a sound way. Data analysis is fundamental in any sequencing project; nevertheless, the hypotheses that are tested are generally biological in nature. The computational results, therefore, must be interpreted to ensure that they are biologically sensible.

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