R PLASMID TRANSFER
OF ANTIBIOTIC RESISTANCE
IN A WASTEWATER TREATMENT PLANT

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ABSTRACT

Enteric bacteria were examined for ability to transfer antibiotic resistance in a wastewater treatment plant. Resistant Salmonella enteritidis, Proteus mirabilis, and Escherichia coli were isolated from clinical specimens and from primary (1°) sewage effluent. Resistance to ampicillin (Ap), chloramphenicol (Cm), streptomycin (Sm), sulfadiazene (Su), and tetracycline (Tc) was demonstrated by spread plate and tube dilution techniques. Plasmid mediation of resistance was shown by acridine orange, dodecyl sodium sulfate, and ethidium bromide curing, and by direct cell transfer. Each donor was mated with sensitive E. coli and Shigella sonnei. Mating pairs (and recipient conrols) were suspended in unchlorinated, 1° effluent that had been filtered and autoclaved. Suspensions were added to membrane diffusion chambers which were then placed in the primary (1°) and secondary (2°) settling tanks of the wastewater treatment plant. Resistant recombinants were detected by replica plating nutrient agar master plates onto XLD agar plates that contained either 10 μg Ap, 30 μg Cm, 10 μg Sm, 100 μg Sy or 30 μg Tc per ml of medium. Mean transfer frequencies for laboratory matings were 2.1×10^{-3}, while in situ matings for 1° and 2° settling were 4.3 and 7.5×10^{-5}, respectively. These values revealed that a significant level of resistance transfer may occur in wastewater treatment plants. Epidemiological significance was discussed.

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INTRODUCTION

The advent of antimicrobial agents provided an extremely useful tool for the prevention and treatment of infectious disease. Erhlich's discovery of selective dye toxicity toward bacterial cells (19) initiated this breakthrough. Then, in the 1920's, Fleming observed the bactericidal characteristics of a Penicillium by-product for gram-positive bacteria. These two observations truly revolutionized medical treatment and expedited the discovery of many antibiotics in the 1940's and 1950's.

It was during this antibiotic boom that Lederberg and Tatum initially demonstrated conjugal transfer of genetic material between bacteria (106). This genetic material was shown not to be associated with the bacterial chromosomes (78), and Jacob and Wollman (90) designated the extra-chromosomal DNA an "episome". The observation of Lederberg and Tatum was linked to antibiotic resistance in 1959, when such resistance among Shigella strains was shown to be transferable during an epidemic of shigellosis in Japan (121). The genetic element was episome-like and called "R-factor" by Mitsuhashi (115).

In the past 20 years, in vitro transfer studies have repeatedly demonstrated intra-specific, intra-generic (16,18), and inter-generic antibiotic resistance transfer (45,46,84,118). Even strains of different families have exhibited in vitro transfer (17,81,102,137). All of these in vitro studies have revealed the relative ease with which bacteria confer their antibiotic resistance to sensitive strains.

Since 1967, transfer studies conducted in living animals (i.e., in vivo) and under natural, environmental conditions (i.e., in situ) have been done to determine the nature and frequency of resistance transfer between natural populations of recipient and donor bacteria. In vivo studies were conducted in laboratory mice (61,82,84,103,153,170,201), sheep (172, 173), pigs (22, 111) and man (84,201,12). These in vivo studies revealed a

decreased transfer frequency in living systems with respect to those observed in vitro.

Published literature contains a single example of in situ R plasmid transfer. Grabow et al. (68) showed transfer of antibiotic resistance among river flora contained in dialysis bags. All other environmental studies have simply demonstrated the presence of resistant and resistance-transferring bacteria in rivers (69,83,92), sea water (36,55), sediments (63,99) and sewage (36,57,66,67,70,126,179,180), not their ability to actually transfer such resistance.

Since bacteria possessing R plasmids readily appear in association with man and other animals, it is unusual that little knowledge of resistance transfer in wastewater treatment has accumulated. Consequently, this study was undertaken to discover the extent of resistance transfer in a wastewater treatment plant. Accordingly, the following objectives were set:

1. to demonstrate antibiotic resistance transfer between hospital and sewage isolates of enteric bacteria contained in a wastewater treatment plant,
2. to determine the frequency of antibiotic resistance transfer among the isolates, and
3. to demonstrate plasmid mediation for all transferable resistance observed.

LITERATURE REVIEW

Introduction

Antibiotics have presented medical practitioners with a highly effective arsenal for treatment of infectious disease. These compunds have generally had devastating effects on the microbial community to which they were exposed. However, some bacteria have demonstrated ability to "spontaneously" develop resistance to these antimicrobial agents; and such spontaneous development has been attributed to mutation. Other means by which bacteria may obtain resistance to antibiotics are by viral transduction, and by transformation. These genetic alterations may be retained because of ecological advantage confered on the host cell, thus allowing it to remain functional while other members of the microbial community are extremely restricted or simply become extinct. These genetic permutations are extremely random and probably contribute little toward the development of a resistant bacterial community in the natural environment (47,177). However, in the past 40 years, it has become evident that bacterial conjugation is a non-random mechanism which allows preferential transmission of beneficial characteristics among bacterial populations.

F Factor

The initial observation of bacterial conjugation by Lederberg and Tatum (106) provided microbial geneticists with a useful model for describing many observed heritable characteristics. These genetic traits were shown to be infective for bacterial populations, given the proper selective environment. Tatum and Lederberg (187) demonstrated the necessity of cell-to-cell contact for conjugal transfer of genetic material between bacteria.

In 1952, Lederberg, et al. (108) described a transferable segment of genetic material that mediated transfer of extra-chromosomal DNA. This

"fertility factor" and other extra-chromosomal segments of DNA were later designated "plasmids" by Lederberg (107). Cavelli (23) demonstrated the integrated state of the fertility (F) factor, calling it 'Hfr" (High frequency recombinant) due to its increased rate of chromosomal transfer (i.e., recombination) during bacterial matings. Hayes (79) showed the rate of recombination among Hfr x F^- (recipient bacteria lacking the F factor) crosses to be several thousand times that observed among F^+ (donor bacteria possessing the F factor) x F^- matings. However, the number of recombinants becoming donor cells in Hfr x F^- crosses was far less than was seen in F^+ x F^- matings (24,78,108). The mechanism for this decreased donor conversion among Hfr cells was made clear by Wollman and Jacob (203,204) in their famous interrupted mating experiment. They found that violent aggitation of the mating mixture at variable times during the experiment resulted in transfer of different amounts of the host chromosome, depending upon the time into the experiment. It was noted that the F factor was the last element transferred between donor and recipient. This accounted for the decreased recipient conversion rate to donor strains when compared to F^+ donors. Jacob and Wollman (90) coined the term "episome" for these small segments of DNA that demonstrated the ability to integrate into promote transfer of the bacterial genome.

Generally, association of the F factor with the host chromosome is a stable integration, forming Hfr cells. Excision of the episome by host-specified nucleases may occur without misreading the host chromosome, resulting in an excised F factor or F^+ cell. The process, called sexduction (80), was initially described by Adelberg and Burns (5,6). The resulting extra-chromosomal fragment, called F', contains both plasmid and chromosomal DNA fragments. The F' strains generally do not become donor bacteria. Instead, these strains are considered "intermediate donors" (80). The source of

the genetic capabilities for this transfer was demonstrated by Adelberg and Burns (6). They revealed that the F factor was responsible for conjugation by infecting F' cells with the F factor from an Hfr strain. This integrated form of F returned conjugal transfer capabilities to the F' cell.

The means used for transfer or genetic elements between bacteria during conjugation is called a sex pilus or "fimbrium" (4). The genetic material coding for this protein tube is associated with the F factor. The molecular mechanism of the transfer has yet to be fully elucidated, but the lumen of the pilus is thought to serve as a passage-way for transport of genetic material (i.e., plasmid or chromosome) between donor and recipient cells. The extremely fragile nature of the pilus is demonstrated by the ease with which shearing occurs during vortex or blender agitation (204).

Those initial studies concerning the fertility factor provided a firm background from which the study of transferable resistance might proceed. Antibiotic resistance was demonstrable from the beginning of antibiotic use. Maclean et al. (111) demonstrated resistance to sulfonamides and Abraham et al. (3) showed penicillin resistance among bacterial populations in the early 1940's. These observations led many people to believe Ehrlich's magic bullets (i.e. antimicrobial agents) were not as bactericidal as once had been proported. In 1956, Kitamoto et al. (98) demonstrated a multiply-resistant strain of Shigella flexneri. This bacterium, isolated from a dysentery patient, showed resistance to chloramphenicol, streptomycin, sulfonamide, and tetracycline. Implications of these observations were not realized

until 1959, when transferable antibiotic resistance to the same antibiotics was observed by Ochiai et al. (134) and Akiba et al. (7) in Japan, among the same strain of bacteria. Shortly thereafter, Datta (40) demonstrated multiple resistance transfer among strains of Salmonella typhimuriumin Great Britain. The transmissible agent conferring resistance was linked to the F factor due to its infective transfer among bacterial populations. In 1960, Mitsuhashi (117) used the term "R-factor" to describe the genetic element that conferred both antibiotic resistance and the ability to transfer the resistance to other bacteria. Mitsuhashi et al. (118) showed the absence of R-factor transfer when cultures were filtered or among filterable agents (i.e., transduction or transformation) and to be dependent on cell-to-cell contact. Although these characteristics were similar to F, a relation between the two plasmids was disproven because F^- and Hfr strains both transmitted antibiotic resistance to species of Shigella in mixed culture (118).

Molecular Nature of R Plasmids

R-factors, as described by Mitsuhashi (117), are conjugative plasmids consiting of two distinguishable component: the resistance transfer factor (RTF or Tra, 132) and resistance (r) determinant. These two components are readily discernable and may be separated differential centrifugation (10,32). The RTF is known to direct transfer of plasmids to which it is covalently bonded, other non-related, unattached plasmids, as well as non-conjugative plasmids. The r-determinant possesses the genetic components for resistance to a variety of bactericidal factors (e.g., heavy metals, ultra-violet radiation, antibiotics) (121)

but may not mediate its own transfer (153,166). The "conjugate" or combined form of these components is generally the form observed among species of Enterobacteraceae (10,32) especially among strains of Escherichia coli, Serratia marcescens, and Salmonella typhimurium (32, 35). The nontransmissible or r-determinant of the R plasmid is commonly seen among strains of Staphylococcus aureus (34) and other bacterial species showing transferable resistance due to viral interaction (15,86) and transformation (37,127). Molecular weights of the two plasmid types reflect the difference between conjugate R plasmids (60×10^6 or 60 Mdal) (10, 32,35,153) and the r-determinant (5-10 Mdal) (34). The difference between the two weights is attributable tothe presence (or absence) of RTF.

Multiple r-determinants associated with a single RTF have been reported (140,141,153). This "transition" (153) from one r-determinant: one RTF state is induced by an increase in the selective nature of the growth medium (e.g., increased antibiotic concentration). The increased number of r-determinants is apparently accomplished by autonomous replication of the r-determinant. Generation of these tandom multimeric r-determinants is initiated by cleavage of the conjugate R plasmid into its constituents. The separated r-determinant is then duplicated, resulting in a head-to-tail arrangement of the "poly r-determinant". The majority of these multimers reassociate with the RTF. However, dissociated multimeric units have been demonstrated among strains of Proteus mirabilis (141). With decreased selective value (i.e., antibiotic concentration), the multimeric r-determinants are systematically deleted from the conjugate plasmid (152). The reason for reversion from polymeric

to monomeric states is thought to be due to homology existing among the genetic elements of the polymeric determinant, itself. THis homology results in competition for the replicating mechanism at the cytoplasmic membrane (32,153). This competition would result in loss of plasmids requiring continuous or stringent replication. However, those plasmids exhibiting relaxed replication would not be lost to the bacterial population (i.e., diluted out).

The replication of R plasmids is known to occur during the S phase of bacterial growth (32,153). THe plasmids are generally found in a covalently-closed, circular state (ccc). However, during the logarithmic phase of the bacterial growth cycle the plasmid associates with the cell membrane and particularly loses its supercoiled nature with selective regions becoming "open-circular" (oc) (32,138). The loss of the supercoiled nature is attributed to the association of the plasmid with specific proteins, thereby forming a "relaxation complex". Clewell and Helinski (29) showed that proteins of the relaxation complex were responsible for the site-specific denaturation which is demonstrated by treatment of purified extracts with protein-ases and detergents. THe protein-specific inhibitors resulted in loss of the oc form and maintenance of ccc. Following site-specific denaturation, the plasmid replicates at the attachment site on the cell membrane (151). The association of the plasmid with its site on the cytoplasmic membrane is called the "unit of segragation" (89). At this site, the plasmid replicated in a manner not unlike the main genome.

Generally, the number of individual plasmid copies is constant between bacterial species (151). This is usually determined by comparing the molecular weight of the plasmid with that of the host chromosome (30,

138). Rownd and Womble (151) have demonstrated the number of specific R plasmids per chromosome to be approximately the same in Escherichia coli, Proteus mirabilis, Salmonella typhimurium and Serratia marcenscens.

However, differences in the number of R plasmids have been shown to occur by means of three different mechanisms. The normal replication of genetic material (chromosome or plasmid) occurs during the exponential phase of bacterial growth. Some species of bacteria have shown the ability to produce multiple copies of their plasmids, even during stationary phase (157,149). This post-log phase replication dramatically increases the plasmid to chromosome ratio. Another method to increase resistance among bacterial populations is to accelerate DNA replication during normal replication. Bacteria exhibiting this characteristic are known as ROR (round of replication) mutants (151). By increasing duplication rates, these individuals have shown the ability to produce several times the normal number of plasmids in any one cell. The other mechanism is by specialized replication of the r-determinant portion of the conjugate plasmid, as described above. THese three mechanisms give rise to increased resistance in the host bacterium due to the gene-dosage response (i.e., 1 gene: 1 protein).

Compatibility

The presence of multiple copies of the same or similar plasmids are seen only in mutant cells or "wild-type" cells in highly selective environments. Without the presence of high antibiotic concentrations, the characteristics seen among bacteria with multiple plasmid copies are lost (112). The persistence of multiple R plasmids of the same type require a high level of plasmid replication.

This energy-requiring process would not be selected for in cells possessing fixed numbers of replication sites and materials (151,74). Infrequent replication results in the eventual dilution of multiple copy conjugates, leading to maintenance of only one or a few copies per cell.

The concept of immunity with respect to elimination of similar plasmids (i.e., molecular nature) was introduced by Watanabe et al. (193). Bacteria possessing specific R plasmids which inhibit F factor acceptance from F donor strains were designated $\underline{fi}^+$ (fertility inhibition). Those R plasmids not losing the F factor following conjugation were considered $\underline{fi}^-$.

The inhibition of fertility has been demonstrated among Hfr and F^+ strains possessing specific R plasmids (74,125,192). In these studies, donor and recipient recombination was repressed by as much as 10^{-5} times the frequency seen among donor cells not harboring the R plasmid. Also, R plasmids showing similar molecular structure have resulted in the competitive exclusion of one or both plasmids when superinfecting a host.

Two theories have arisen regarding the inability of bacteria with specific R plasmids to accept and maintain infecting R plasmids. The first one regards the exclusion of the infecting plasmid and/or resident plasmid due to the molecular similarity of the two. Echols (49) first showed this imcompatibility among the R plasmids of enteric bacteria. In his paper, Echols postulated the presence of specific replication mechanisms in limiting numbers. When the bacterium was "superinfected" (74) with a similar plasmid, competition for the replicating mechanism resulted in loss of one or both R plasmids.

The other theory involves specificity of the pilus for sites on receptor cells (24,72). The pilus, a translation product of the resident plasmid, shows a high degree of specificity for recipient cells. This has been demonstrated by the specificity of filamentous bacteriophage for the attachment sites on the pilus. However, presence of specific plasmids in the recipient cells results in resident-specified alterations of these receptor sites, making the recipient unavailable for conjugation and therefore, R plasmid transfer (103). The selective acceptance of conjugative plasmids by bacteria possessing similar resident plasmids is most likely a combination of the two mechanisms.

The use of plasmid compatibility has been suggested as a tool for epidemiological study of R plasmids in natural environments to selectively identify resistance patterns during epidemics of related and nonrelated species (40). The use of this scheme for plasmid identification is limited by the hypermutable nature of plasmids. The presence of multiple determinants in a single conjugate plasmid, insertions, crossovers, transposons, and a variety of mutations, present a multitude of changes that may affect the molecular make up of plasmids. These quantitative and qualitative alterations may or may not result in loss of the infecting R plasmid or "dislodgement" (31) of the resident plasmid. Because plasmid elimination is not strictly adherent to elimination by residents of the recipient bacterium, a definitive result is not always obtained by using compatibility studies.

Several quantitative techniques have been introduced to show the molecular relatedness among R plasmids that confer similar patterns of resistance. Among these are buoyant density centrifugation (142,165)

and sucrose gradient velocity centrifugation (165). These methods are premised upon the differences in molecular weight and in surface area exhibited by plasmid species (199). Studies utilizing these techniques assume that plasmids with similar size are similar or related. Other techniques include vertical and horizontal agarose gel electrophoresis (1,113). These techniques demonstrate the difference in migration distances, in a highly purified gel, according to ionic state and molecular weight. These techniques have been combined with nuclease digestion (153,199). Together, these techniques provide a highly specific separation of similar plasmid segments. One of the most sensitive techniques for relatedness studies is heteroduplex analysis (91). This method utilizes DNA segments of known composition to measure the degree of association between plasmids. Segment association is dependent on base pair homology between strands. The degree of annealing between strands is commonly visualized by using an electron microscope but absorbance at 260 nm is also used. Together, these techniques have provided specific characterizationof the molecular nature and relatedness of R plasmids.

Resistance Mechanisms

The nature of the inhibitory effect of an antibiotic is specific to that antibiotic. The therapeutic value of each individual agent is dependent upon the narrow spectrum of activity shown by that antibiotic and upon the extent of the inhibitory effect demonstrated on the infectious organisms being treated.

The classification of these compounds has presented problems for many years. Proposals have been submitted for classification on the basis of chemical structure (162,203), origin (133) and mode of action (60).

Among the clinically useful antibiotics that are specific for bacteria, the spectrum of activities are divided between those inhibiting cell wall synthesis (e.g., penicillin, cycloserine, bacitracin) and those inhibiting protein synthesis. This latter group is divided among agents inhibiting bacerial transcription (e.g., rifampin), or translation (e.g., tetracycline, aminoglycosides) or peptide release from the ribisome (e.g., chloramphenicol, macrolide antibiotics).

These compounds for a time provided a useful means for the control and treatment of infectious disease. However, indiscriminant use of these compounds, such as for prophylaxis and for growth promotion in animal husbandry (12,92) have probably provided a selective environment for the occurrence and proliferation of antibiotic resistance.

The origin of plasmids bearing resistance conferring genes in association with the ability for self-transmission have been the subject of much conjecture since Akiba and Ochiai first demonstrated transferable resistance. Generally, the ultimate source of antibiotic resistance is considered to be organisms from animals and/or humans (118). However, resistance transmission was initially demonstrated in Japan, but other resistance-transferring bacteria were found in Taiwan and Israel during the same year. These observations led Mitsuhashi (122) to believe that the resistance plasmids had not been transported by world travellers, but had arisen separately, in different parts of the world.

The molecular nature of the original R plasmid is thought to have been the result of a recombinant event. The presence of chromosomally-mediated resistance is widely documented in the literature (163). Bacteria, possessing the resistance genes, retain their susceptibility to bacteriophage infection. The phage genome may integrate into the bacterial

chromosome. The result of misreading of the genetic markers for excision of the phage genome may have resulted in exchange of a segment of bacterial chromosome for a portion of the phage genome. Furthermore, this exchange may have resulted in removal of bacterial resistance genes (195). Misreading during excision is not uncommon among bateriophage systems or in relation to the F factor (i.e., sexduction) (118). These genetic interactions, along with the use of sulfonamides, streptomycin tetracycline, and chloramphenicol from 1945 to 1950, probably accounted for the appearance and establishment of the first transferable antibiotic resistance (i.e., R plasmid) (122).

The mechanism of resistance to the more commonly used antibiotics is highly variable. However, the R plasmid mediated mechanisms are usually one of two general types (163):

1. enzymatic alteration of the antibiotic, resulting in compounds with decreased bactericidal activity,

or

2. physical alteration of the bacterial components, leaving the bacterium less susceptible to the effects of the antibiotic.

R plasmid-mediated resistance to the penicillins is widely documented (110,121,163,179). The major activity of these resistance plasmids is the production of a β-lactamase, which hydrolyzes the amide bond of the β-lactam ring, common to pencillin and penicillin-like molecules. This hydrolysis prevents the penicillin from functioning as a substrate for the enzyme responsible for severing the terminal alanine of the pentapeptide in peptidoglycan synthesis. Curtis et al. (36) have reported an R plasmid-mediated resistance to penicillin <u>not</u> due to β-lactamase activity. Unfortunately, the mechanism has not yet been determined.

Resistance to aminoglycosides has been shown to be primarily enzymatic in nature. Three classes of enzymes have been implicated as reasons for aminoglycoside inactivation: acetyl-transferases, adenyl-

transferases and phosphotransferases. The heterocyclic aminoglycosides present multiple sites for addition of various deactivating groups by the aforementioned enzymes (121,140). Resistance demonstrated among aminoglycosides is usually cross-reactive between several members of this group (18,47,124,140). Cross-reactivity is attributable to the molecular structure exhibited by aminoglycosides and to the relatively broad specificity exhibited by the individual transferases.

Chloramphenicol resistance has been associated with both enzymatic deactivation and decreased drug permeability. The primary mechanism of enzymatic degradation is through the activity of acetyl-transferases. Two major sites of activity have been shown susceptible to enzymatic attack in the aliphatic portion of the chloramphenicol molecule. The enzyme, chloramphenicol acetyl-transferase (CATase), has been demonstrated to mediate the formation of both 3-O-acetyl chloramphenicol and 1,3-O-diacetyl chloramphenicol (56,163). Permeability alterations have been shown to reduce the susceptibility of strains of Escherichia coli to chloramphenicol (125). The barrier is thought to be either in the cytoplasmic membrane or cell wall. The altered pentration has been attributed to a specific R plasmid, designated R_{MS70}. Mitsuhashi et al. (121) have also revealed decreased chloramphenicol assimilation in Pseudomonas aeruginosa due to R plasmid, R_{KR102}.

Resistance to tetracyclines appears to be due to decreased chemical transport into the cell. Izaki et al. (86,87) demonstrated tetracycline accumulation in the external environment of cells possessing resistance to the agent. They also showed the system to require an energy source and magnesium ions, and they demonstrated soduim azide sensitivity (inhibitor

of oxidative phosphorylation). These factors led them to postulate the presence of an inducible enzyme system responsible for the production of a tetracycline transport inhibitor in the periplasmic space. The production of inhibitor was shown to be susceptible to chloramphenicol treatment (163), thereby indicating its proteinaceous nature. This inhibitor was called "TET protein" by Levy and McMurray (107) and they demonstrated its presence in association with tetracycline resistance plasmid, R_{222}.

Sulfonamide resistance has been shown to be due to synthesis of an enzyme not susceptable to sulfonamide inhibition and to lowered sulfonamide permeability (121). In cells susceptible to sulfa drugs, dihydropterate synthetase is inhibited by the presence of the drug. This enzyme mediates the conversion of p-aminobenzoglutarate, dihydropteridine and p-aminobenzoate to dihydrofolate, a precursor of tetrahydrofolate (folic acid). Wise and Abou-Donia (198) have isolated an R plasmid coding for dihydropterate synthetase showing no susceptibility to sulfonamides. Akiba and Yokata (8,9) have shown decreased uptake of sulfonamide by bacteria possessing specific R plasmid while having a totally sensitive dihydropterate synthetase. The mode of resistance seems to depend on which R plasmid is present.

These mechanisms reveal the variety of antibiotic resistance mechanisms available to bacterial populations. Although many of the mechanisms are similar (e.g., transfer of acetyl, adenyl or phosphate groups), the enzymes mediating these reactions are very specific as to the groups or type of compound which it will chemically alter to a non-inhibitory form. Similarly, cell permeability to these inhibitory agents is extremely specific for the various cellular systems. However, the site of altered

permeability or the mode may be quite similar from one antibiotic to the next. Together, these resistance mechanisms have provided an efficient means for bacteria to survive in environments altered to remove the non-resistant strains.

Epidemiology

The incidence and transmission of R plasmids among bacterial communities has created a major concern among public health officials for the control of infectious disease. Although R plasmids are commonly associated with virulent bacteria, resistant strains have been shown to be ubiquitous among the normal flora of healthy individuals (199). Datta (42,43) and Sogaard (175) demonstrated similar resistance patterns among the flora of hospitalized patients before their admission. They also demonstrated similar resistance patterns among nonhospitalized and hospitalized patients. Because R plasmids are prevalent among admitted patients, an increase in antibiotic resistance among nosocomial isolates has been documented in the last 15 years (21,64). Turck (193) revealed this high level of resistance to be partially accountable for the increased incidence of infectious disease due to gram-negative bacteria. Isenberg and Berkman (87), Kawakami et al (96), Salzman et al. (159), Thoburn et al. (187), and Aandahl (2) demonstrated the incidence of transferable resistance among strains causing nosocomial disease. These bacterial isolates generally showed higher minimum inhibitory concentrations (MIC) than infectious bacteria recently associated with human disease in a nonhospital environment. Mouton et al. (125) and Valtonen et al., (192) showed similar results among patients receiving prophylactic doses of tetracycline, kanamycin, and ampicillin. These studies also noted rapid conversion of the resident flora to a resistant state corresponding

to the administered tetracycline, kanamycin and ampicillin antibiotics. Lacey (103) implied that resistance transfer was infrequent or rare in vivo. However, Egawa et al. (53), Gardner and Smith (61), Laufs and Kleiman (104), Zidmundova et al, (207), Witchitz and Chabbert (201), Jobanputra and Datta (93), Schroter and Humpfner (163), Traub et al. (190), and Curie et al. (37) presented surveys among hospitalized patients. Their studies showed identical resistance patterns and similar plasmids among strains of Escherichia coli, Proteus species, Serratia marcescens, Enterobacter species, Klebsiella species, and Pseudomonas aeruginosa. All of these infectious were of nosocomial origin.

Demonstration of in vivo acquisition of antibiotic resistance within a human being was shown by Kayser et al. (97) in a kidney allograft recipient. Because of massive doses of immuno-suppressive therapy used to retard graft rejection, the individual was left with only parentally-administered antibiotics to combat a nosocomially acquired Salmonella dublin infection. With lowered host defenses, the individual also acquired a Klebsiella infection. The Klebsiella transferred its aminoglycoside and β-lactamase activity to the Salmonella. This was revealed when both organisms showed identical resistance patterns upon isolation.

The incidence of R plasmids among common sources of urinary tract infection (50,72,127,138,149,207), diarrheagenic disease (2,11,139,179) and respiratory infections (37,61) have led some health officials to believe in the existance of a reservoir of R plasmid-carrying bacteria among the animal community, leading to transfer of resistance among bacteria in the human population. Hartley et al. (77) and Smith (168) demonstrated the occurrence of similar resistance patterns among animal and human isolates of the same serotype. Therefore, it was not unusual

that transferable resistance has been demonstrated in the flora of domestic dogs (60), pigs (22,111,180), cattle (77,143,160), wild birds (27,161), toads and lizards (101).

From a global standpoint, demonstration of transferable resistance by Akiba and Ochiai barely scratched the surface of R plasmid transfer. Nakaya et al. (127) and Akiba et al. (7) demonstrated resistance transfer among enteric bacteria other than the E. coli-Shigella flexneri system they initially used to demonstrate multiple resistance. Datta (40) in 1962 demonstrated transferable resistance among strains of Salmonella typhimurium in a hospital epidemic in England. Since that time, transferable resistance has been shown among Salmonella and Shigella in Korea (28,29), Nepal (186), India (95), Romania (154), Czechoslovakia (162), Italy (157), the Netherlands (195), Algeria (145), South Africa (199), Mexico (136,45), the United States (128), and Japan (181,182,183,184). The widespread nature of resistance has led to the utilization of computer analysis to allow better understanding of resistance patterns on a broad scale (73,100).

It has been proven that there are several modes of transmission for resistance among bacteria in biotic and abiotic environments (148). In the biotic community, transmission is generally attributed to the consumption of infected foods (193) or other materials contaminated with fecal matter. The abiotic transmission of resistance, however, is commonly associated with septic tanks (146) or sewage treatment (148). The incidence of transferable resistance among the flora in wastewater has been demonstrated by Sturtevant et al. (178,179), Fontaine and Hoadley (57) and Grabow et al. (64,65,70). Although the total number of bacteria is significantly decreased by wastewater treatment, resistant bacteria are readily demonstrable at the sewage treatment plant outfall (67,69).

The high incidence of these resistant forms among the flora of hospital effluents (57,110) has presented public health officials with a major task for control of resistance dissemination both in and beyond the treatment plant.

R plasmid transfer has been shown in situ in a river below a sewage treatment plant by Grabow, et al. (69). This observation is especially significant because enteric pathogens with high levels of resistance, have been known to cause outbreaks of dysentery below sewage outfalls (25,150).

Transferable resistance, however, has yet to be demonstrated in situ among the normal flora within the wastewater treatment plant. In regard to the high-level of resistance associated with some of the enteric pathogens found in the treatment plant, resistance transfer could occur if the environment proved hospitable to the cell-to-contact required for transfer. However, the aquatic environment within the treatment plant is purposefully made as inhospitable as possible to facilitate removal of the pathogenic bacteria present in the primary effluent. Therefore, to help resolve this paradox, an in situ study was conducted to demonstrate transferable antibiotic resistance among the natural flora of the wastewater treatment plant.

MATERIALS AND METHODS

Site Description

In situ resistance transfer studies were conducted in the primary and secondary water clarifiers of the La Crosse Wastewater Treatment Plant, La Crosse, Wisconsin. The activated sludge method for treatment of approximately 1.7 million gallons per day is utilized at the treatment plant. Monthly averages for pH, suspended solids (S.S.) removal, per cent biochemical oxygen demand (B.O.D.) removal, dissolved oxygen (D.O.) concentration, sludge volume index (S.V.I.) for the treatment plant in 1979 are listed in Appendix 1.

Isolation Techniques

Bacteria were isolated from sewage effluent by using standard enrichment culture techniques (175). GN and Tetrathionate broths (Difco Laboratories, Detroit, Michigan) served as selective enrichment media for isolation of Shigella and Salmonella species. Approximately 0.5 L of the primary effluent was filtered through a type HC filter (HCWG-047-00, Millipore Corporation, Bedford, Massachusettes). The filter was then placed in 10 ml GN or Tetrathionate broth. The Tetrathionate tubes were incubated at 35° C for 24 h, while GN tubes were incubated at 41.5° C for 18 to 24 h. Broths showing noticeable turbidity were streaked onto XLD agar (Difco) plates for isolation and preliminary identification. Isolated colonies demonstrating colonial characteristics typical of either genus were identified as outlined by Edwards and Ewing (52).

Proteus species and Escherichia coli isolations were facilitated by serial 1:10 dilution of wastewater samples in 0.85% (w/v)NaCl. Countable plates (30-300 colonies) were obtained by spread plating 0.1-ml

aliquots onto XLD plates. Typical colonies were selected and identified by methodology previously cited (52). These methods provided isolations of Escherichia coli (two strains), Proteus mirabilis (one strain) and Shigella sonnei (one strain) from primary effluent. Standard biochemical set reactions for the organisms are listed in Appendix 3.

Clinical isolates of Salmonella enteritidis (one strain), Proteus mirabilis (one strain) and Escherichia coli (three strains) were acquired from the bacteriology laboratory of La Crosse Lutheran Hospital, La Crosse, Wisconsin. With the exception of S. enteritidis, these isolates were of nosocomial urinary tract origin. S. enteritidis was obtained from a patient with gastroenteritis. Preliminary identification was conducted by hospital personnel using the API 20E system (Analytab Products, Plainview, New York). Their identification was confirmed by using techniques previously cited (52). Stock cultures of hospital and sewage isolates were maintained at room temperature (20°C) in Motility Test medium (85) and in a frozen state (-70°C) in Brain Heart Infusion broth (Difco).

Antibiotic Resistance

All bacterial isolates were assayed for resistance to five antimicrobial agents: ampicillin trihydrate (Ap), chloramphenicol (Cm), streptomycin sulfate (Sm), 2-sulfanilamidopyrimidine and tetracycline hydrochloride (Tc). Sources of the chemicals were as follows: Ap and Su (United States Biochemical Corporation, Cleveland, Ohio) and Cm, Sm and Tc (Calbiochem-Behring Corporation, La Jolla, California). Stock 125-ml quantities of each compound (1-4 mg/ml) were filter-sterilized and refrigerated (4°C). Appropriate quantities of Cm, Sm and Tc were suspended in distilled water. However, it was necessary

to dissolve Ap in 10% (v/v) methanol, and Su was dissolved in 0.1 N NaOH.

Antibiotic resistance was demonstrated by using tube dilutions in Nutrient broth (NB) (Difco). Bacteria were grown in 10 ml NB for approximately 6 h. One ml of each culture (200-500 cells/ml) was transferred to 9-ml NB blanks. The broth tubes contained antibiotics in concentrations of 10 μg/ml, 30 μg/ml, 50 μg/ml, 100 μg/ml, 500 μg/ml, 1000 μg/ml, 1500 μg/ml, and 2000 μg/ml. Tubes were then incubated at 35°C for 18 h. Growth was determined by spectrophotometric absorbance at 620 nm (Spectronic 20, Bausch & Lomb, Rochester, New York).

Growth Curve and Optical Density Determinations

Growth curves were determined for each bacterial species growing in NB or sewage effluent (Appendices 4,5,6,7,8, and 9). The sewage effluent was prefiltered with type AP prefilters (AP25-047-00, Millipore); this filtrate was then passed through type HC filters to produce a secondary filtrate. The secondary filtrate was autoclaved and designated "Sterile Sewage" (SS). This medium (SS) was of sufficient transparency to be used for spectrophotometric determinations for bacterial enumeration. Absorbance at 620 nm was determined for each SS culture (Spectronic 20, Bausch & Lomb).

To initiate the growth curve experiments, 24-h NB "seeds" were added to the growth curve media (either NB or SS). NB seed cultures were visually standardized against a McFarland Standard No. 5 (1.5×10^9 bacteria/ml) (Difco) with 0.85% (w/v) NaCl as the diluent. Cultures were then inoculated into 100 ml of medium (NB or SS) and incubated at 20°C for the duration of the determination. Bacterial cultures were then enumerated at 0.5-h intervals by absorbance at 620 nm and by spread plating 0.1 ml of appropriate dilutions (10^{-3}, 10^{-4}, 10^{-5})

onto Nutrient agar (Difco) plates. Cell counts were plotted as a function of absorbance to obtain a standard curve. These curves were used to determine inoculum densities for resistance transfer studies.

Transfer Studies

Resistance transfer studies were conducted both in vitro and in situ. In vitro studies were completed in both NB and SS; in the laboratory; in situ studies were conducted with only SS at the treatment plant.

In vitro transfer frequencies were determined by using 20 ml of medium at 20°C. Approximately 2.9×10^6 resistant (donor) and sensitive (recipient) late log phase bacteria of different species were inoculated into the growth medium (NB or SS). Transfer studies were conducted for 3 h. At 1 h intervals 0.1-ml samples were removed and diluted in 0.85% (w.v) NaCl. Appropriate samples were then spread-plated onto Nutrient agar plates. Countable plates (30-300 colonies) served as the standard inoculum for replica plating onto XLD agar plates containing antibiotics. Antibiotic concentrations used were: Ap, 10 μg/ml; Cm, 30 μg/ml; Sm, 10 μg/ml; Su, 100 μg/ml; or Tc, 30 μg/ml (57). Donor and recipient cells were differentiated on the basis of colonial morphology, fermentation and hydrogen sulfide production on XLD. Confirmation of colonies identified in this manner was accomplished with API 20E test strips (Appendices 10-14).

In situ transfer studies were completed in both primary and secondary clarifiers during the week of 2-9 October 1979. (Treatment plant parameters for this period are listed in Appendix 2). Modified Stuart-McFeters membrane-filter diffusion chambers (56) were constructed and used as the transfer vessel for in situ studies. Modifications involved increasing the central spacer to allow increased volume within the

chamber (from 20 to 30 ml), and the addition of plastic mesh over the surface of the membranes. Each chamber was fitted with a 0.4-μm polycarbonate membrane (No. 111507, Nucleopore Corporation, Pleasanton, California) and then was ethylene oxide sterilized. Chambers were allowed to equilibrate with the aquatic environment of the treatment plant for approximately 0.5 h followed by inoculation with a 20-ml suspension of 2.9×10^6 donor and 2.9×10^6 recipient bacteria each suspended in SS. In situ studies were conducted for 3 h. At 1 h intervals, 0.1-ml samples were removed and diluted in 0.85 (w/v) NaCl. Resistance and bacterial identification of each recipient was demonstrated as described for the in vitro studies.

Transfer frequencies (TF) for the individual antibiotics in each mating were determined by the following formula:

$$TF = \frac{\text{number of recombinants}}{(\text{number of donors/ml})(\text{dilution fractor})(\text{ml of inoculum})}$$

Mean TF and differences between TF were statistically evaluated using a special modification of the student's T-test (174).

Transduction and Transformation

The possibility of transformation and/or viral transduction mediating antibiotic resistance transfer in the wastewater treatment plant was determined by placing control membrane-diffusion chambers inoculated on with antibiotic-sensitive (recipient) bacteria into the clarifying tanks. Chambers were inoculated with 4×10^{12} bacteria; this large inoculum permitted detection of recombinant rates with a minimum frequency of 10^{-11} per recipient. No steps were taken to distinguish between the two recombinant mechanisms, either transformation or transduction.

Plasmid Curing

Resistance curing experiments were complete on late logarithmic phase donor and recombinant bacterial populations. The curing agents were: 25 and 50 μg/ml acridine orange (no. 46005, Eastman Kodak Company, Rochester, New York) (76), 1% dodecyl sodium sulfate (no. 0-2674, Eastman Kodak Company) (4,154), and 10^{-5} M ethidium bromide (No. 331565, Calbiochem-Behring Corporation) (189,48). These compounds were added separately to Nutrient agar plates. Curing was accomplished by spread plating 0.1 ml of a 6-h NB culture (200-500 cells/ml) onto the plate. Cultures were then replica plated onto unaltered (no agent present) Nutrient agar plates, which served as the template for replica plating onto XLD. Antibiotics, to which the organisms had previously shown resistance, were incorporated in the XLD to determine the curing efficiency of each agent.

RESULTS

Antibiotic Resistance

Resistance of Salmonella enteritidis (H), Proteus mirabilis H, Proteus mirabilis S, Escherichia coli H1, Escherichia coli H2, Escherichia coli H3, Escherichia coli S1, Escherichia coli S2, and Shigella sonnei S to Ap, Cm, Sm, Su, and Tc is listed in Table 1. Two of the nine strains tested showed no resistance to the five antibiotics. The totally sensitive strains, Escherichia coli H3 and Shigella sonnei S were used as recipients for transferable resistance from the above resistant strains for all transfer studies conducted both in vitro and in situ.

Among organisms demonstrating resistance to the antibiotics, maximum resistance was seen toward Su ($\bar{x}$ = 2000 μg/ml), followed by Tc ($\bar{x}$ = 1500 μg/ml), Cm ($\bar{x}$ = 1500 μg/ml), Sm ($\bar{x}$ = 566 μg/ml), and Ap ($\bar{x}$ = 100 μg/ml). Furthermore, only multiple drug resistance was observed for the resistant organisms. Of the isolates used in the transfer experiments, those showing resistance to three of the five antibiotics were the most common (71.4%); resistance to two or to four antibiotics were not as prevalent (14.3%).

When comparing hospital and sewage isolates of the same species (e.g., E.coli H1 and H2 with S1 and S2; P. mirabilibis H with S), high-level chloramphenicol resistance was demonstrable only for sewage isolates; streptomycin resistance was more prevalent among hospital isolates. Generally, however, antibiotic resistance to the chemical agents was evenly distributed among strains from both environments.

Table 1. Demonstrable antibiotic resistance

Organism	Antibiotic concentration (ug/ml)				
	Ap[1]	Cm	Sm	Su	Tc
Salmonella enteritidis H[2]	0	0	100	2000	0
Proteus mirabilis H	0	0	100	2000	1500
Proteus mirabilis S	0	1500	100	2000	1500
Escherichia coli H1	100	0	1500	2000	0
Escherichia coli H2	100	0	1500	2000	0
Escherichia coli H3	0	0	0	0	0
Escherichia coli S1	100	0	100	2000	0
Escherichia coli S2	100	1500	0	2000	0
Shigella sonnei S	0	0	0	0	0

[1] Ap = ampicillin trihydrate, Cm = chloramphenicol, Sm = streptomycin sulfate, Su = 2-sulfanilamidopyrimidine, Tc = tetracycline hydrochloride

[2] Strain abbreviations: H = hospital isolate, S = sewage treatment plant isolate

Transfer Frequencies

Resistance transfer was detected as previously described for in vitro and in situ studies. NB or SS served as the growth medium for laboratory studies, while only SS was used in wastewater treatment studies. By nature of their sensitivity (uniform lack of resistance) to the antibiotics which were tested, E. coli H3 and S. sonnei S were used as recipients for detection of transferable resistance. Frequencies for the in vitro and in situ transfer studies are listed in Table 2. S. sonnei S was mated with the seven resistant isolates to detect transfer of resistance. However, to facilitate identifications of donor and recipient strains, the recipient (E. coli H3) was not crossed with resistant E. coli isolates (H1, H2, S1, S2).

In vitro transfer occured more often ($\bar{x} = 2.1 \times 10^{-3}$/donor) than in situ transfers of antibiotic resistance ($\bar{x} = 5.9 \times 10^{-5}$/donor). Mean in vitro transfer frequencies from NB experiments ($\bar{x} = 2.1 \times 10^{-3}$) and SS ($\bar{x} = 2.2 \times 10^{-3}$) were approximately equal. However, closer examination revealed that transfer of resistance did not occur in some SSmixtures. Statistical analysis (F-test, 174) confirmed this observation ($P < .03$).

In order to eliminate other factors, in situ matings were conducted only in the SS medium. After removing this factor, transfer frequencies detected in the primary clarifier ($\bar{x} = 4.3 \times 10^{-5}$) were significantly ($P < .1$) less than those detected in the secondary clarifier ($\bar{x} = 7.5 \times 10^{-5}$).

The transfer studies also revealed a difference in the transfer frequency seen for the two recipients. In vitro and in situ frequencies revealed E. coli H3 recombinant rates ($\bar{x} = 2.7 \times 10^{-3}$) to be higher than those seen for S. sonnei S ($\bar{x} = 1.5 \times 10^{-3}$), when P. mirabilis H, P. mirabilis S and Salmonella enteritidis H were used as donors.

The mating stability of E. coli systems was further emphasized by the relatively small difference observed for the four resistant E.

Table 2. Resistance transfer frequency

Organisms	Ab[1]	Transfer frequency NB[2] (10^{-3})	SS (10^{-3})	1^{o} (10^{-5})	2^{o} (10^{-4})
S.enteritidis H x E.coli H3					
	Sm[3]	5.2	2.9	8.0	2.7
	Su	3.8	3.5	8.0	2.0
S.enteritidis H x S.sonnei S[4]					
	Sm	2.2	2.3	1.7	0.3
	Su	2.7	1.5	1.7	1.0
P.mirabilis H x E.coli H3					
	Sm	2.3	2.7	3.5	0.4
	Su	2.7	2.1	3.5	1.0
	Tc	1.3	0.0	0.0	0.3
P.mirabilis H x S.sonnei S					
	Sm	1.0	0.0	0.0	0.9
	Su	1.0	0.0	6,9	1.2
	Tc	0.7	0.0	0.0	0.0
P.mirabilis S X E.coli H3					
	Cm	0.5	0.0	0.0	0.0
	Sm	3.8	1.4	3.5	1.2
	Su	2.7	0.9	3.5	1.2
	Tc	2.0	0.0	3.5	0.0
P.mirabilis S x S.sonnei S					
	Cm	0.0	0.0	0.0	0.0
	Sm	0.3	0.0	0.0	1.0
	Su	0.5	0.0	3.5	1.7
	Tc	0.5	2.4	3.5	1.3
E.coli H1 x S.sonnei S					
	Ap	1.0	1.9	5.0	0.2
	Sm	2.0	1.4	2.5	0.2
	Su	2.7	1.0	3.8	0.2
E.coli H2 x S.sonnei S					
	Ap	1.8	1.0	5.4	0.1
	Sm	2.8	1.4	4.4	0.1
	Su	0.7	0.7	5.0	0.1
E.coli S1 x S.sonnei S					
	Ap	6.0	4.5	6.7	0.5
	Sm	2.2	1.9	3.7	0.4
	Su	3.0	2.3	5.4	0.3
E.coli S2 x S.sonnei S					
	Ap	2.3	4.3	3.3	0.5
	Cm	1.5	1.7	3.8	0.2
	Su	0.5	3.6	3.2	0.5

[1] Ab = antibiotic; [4] S = sewage isolate, H = hospital isolate

[2] NB = Nutrient broth, SS = sterile sewage, 1^{o} = primary clarifier, 2^{o} = secondary clarifier

[3] Sm = streptomycin sulfate, Su = 2-sulfanilamidopyrimidine, Ap = ampicillin trihydrate, Cm = chloramphenicol, Tc = tetracycline hydrochloride

coli donors mated with the sensitive S. sonnei recipient. Transfer frequencies in primary ($\bar{x} = 4.4 \times 10^{-5}$) and secondary ($\bar{x} = 2.8 \times 10^{-5}$) clarifiers revealed little statistical difference ($P > .25$). These values emphasize the ability of E. coli to serve as a stable donor and dependable recipient of antibiotic resistance for interspecific matings in vitro and in situ.

Curable Resistance

Resistance elimination, using acridine orange, dodecyl sodium sulfate and ethidium bromide, was conducted on resistant donor and resistant recombinant organisms in the manner previously described. Resistance curing was accomplished using 25 µg/ml (Table 3) and 50 µg/ml (Table 4) acridine orange. Generally, the curing effect of the two concentrations was of equal effect. However, only 50 µg/ml acridine orange was able to eliminate Sm and Tc resistance from the recombinant S. sonnei S that had been mated with E. coli H1 and P. mirabilis H, respectively. Resistance elimination by 1% (w/v) dodecyl sodium sulfate (SDS) is represented in Table 5. With the exception of donor P. mirabilis strains, which failed to grow on SDS-Nutrient agar plates, resistance among donor organisms was cured (eliminated) in 57% of the cultures. A similar elimination rate (50%) was observed among the recombinant organisms. Resistance curing by 10^{-5} M ethidium bromide was also conducted (Table 6). Using this technique, much of the resistance exhibited by the recombinants (71.4%) was eliminated, while a smaller percentage (28.6%) of the resistance seen among donor organisms was eliminated by ethidium bromide exposure.

Table 7 summarizes the total resistance curing by all four treatments (i.e., 3 agents). Among the donor organisms, 47.6% of the demonstrable resistance was eliminated by exposure to the curing agents.

Table 3. Resistance curing by 25 ug/ml acridine orange

Organism	Resistance				
	Ap[1]	Cm	Sm	Su	Tc
S. enteritidis H[2]	0[3]	0	+/+[4]	+/+	0
P. mirabilis H	0	0	+/-	+/+	+/+
P. mirabilis S	0	+/-	+/+	+/+	+/+
E. coli H1	+/+	0	+/-	+/-	0
E. coli H2	+/+	0	+/-	+/-	0
E. coli S1	+/+	0	+/-	+/+	0
E. coli S2	+/+	+/-	0	+/+	0
S. sonnei S - E. coli H1[5]	+/-	0	+/+	+/-	0
S. sonnei S - E. coli H2	+/+	0	+/-	+/+	0
S. sonnei S - E. coli S1	+/-	0	+/+	+/+	0
S. sonnei S - E. coli S2	+/+	+/+	0	+/+	0
S. sonnei S - P. mirabilis H	0	0	+/-	+/+	+/+
S. sonnei S - P. mirabilis S	0	+/-	+/-	+/+	+/-
E. coli H3 - S. enteritidis H	0	0	+/-	+/-	0
E. coli H3 - P. mirabilis H	0	0	+/-	+/+	+/+
E. coli H3 - P. mirabilis S	0	+/-	+/+	+/+	+/+

[1]Ap = ampicillin trihydrate, Cm = chloramphenicol, Sm = streptomycin sulfate, Su = 2-sulfanilamidopyrimidine, Tc = tetracycline hydrochloride

[2]Strain designation:S = sewage isolate, H = hospital isolate

[3]0 = no initial resistance, + = resistance, - = no resistance

[4]Pre-curing resistance listed in numerator, post-curing in denominator

[5]Recombinant is listed first, donor from which the recombinant received resistance is listed second.

Table 4. Resistance curing by 50 ug/ml acridine orange

Organism	Resistance				
	Ap[1]	Cm	Sm	Su	Tc
S. enteritidis H[2]	0[3]	0	+/+[4]	+/+	0
P. mirabilis H	0	0	+/-	+/+	+/+
P. mirabilis S	0	+/-	+/+	+/+	+/+
E. coli H1	+/+	0	+/-	+/-	0
E. coli H2	+/+	0	+/-	+/-	0
E. coli S1	+/+	0	+/-	+/-	0
E. coli S2	+/+	+/-	0	+/-	0
S. sonnei S - *E. coli* H1[5]	+/-	0	+/-	+/-	0
S. sonnei S - *E. coli* H2	+/+	0	+/-	+/+	0
S. sonnei S - *E. coli* S1	+/-	0	+/-	+/-	0
S. sonnei S - *E. coli* S2	+/+	+/+	0	+/+	0
S. sonnei S - *P. mirabilis* H	0	0	+/-	+/+	+/-
S. sonnei S - *P. mirabilis* S	0	+/-	+/-	+/+	+/-
E. coli H3 - *S. enteritidis* H	0	0	+/-	+/-	0
E. coli H3 - *P. mirabilis* H	0	0	+/-	+/-	+/+
E. coli H3 - *P. mirabilis* S	0	+/-	+/-	+/+	+/+

[1]Ap = ampicillin trihydrate, Cm = chloramphenicol, Sm = streptomycin sulfate, Su = 2-sulfanilamidopyrimidine, Tc = tetracycline hydrochloride

[2]Strain designation: S = sewage isolate, H = hospital isolate

[3]0 = no initial resistance, + = resistance, - = no resistance

[4]Pre-curing resistance listed in numerator, post-curing in denominator

[5]Recombinant is listed first, donor from which the recombinant received resistance is listed second

Table 5. Resistance curing by 1% SDS

Organism	Resistance				
	Ap[1]	Cm	Sm	Su	Tc
S. enteritidis H[2]	0[3]	0	+/+[4]	+/+	0
P. mirabilis H	no growth				
P. mirabilis S	no growth				
E. coli H1	+/-	0	+/-	+/-	0
E. coli H2	+/-	0	+/-	+/-	0
E. coli S1	+/+	0	+/-	+/+	0
E. coli S2	+/+	+/-	0	+/+	0
S. sonnei S - E. coli H1[5]	+/+	0	+/+	+/-	0
S. sonnei S - E. coli H2	+/-	0	+/-	+/-	0
S. sonnei S - E. coli S1	+/-	0	+/+	+/-	0
S. sonnei S - E. coli S2	+/-	+/+	0	+/-	0
S. sonnei S - P. mirabilis H	0	0	+/+	+/+	+/+
S. sonnei S - P. mirabilis S	0	+/-	+/+	+/+	+/+
E. coli H3 - S. enteritidis H	0	0	+/+	+/+	0
E. coli H3 - P. mirabilis H	0	0	+/-	+/+	+/+
E. coli H3 - P. mirabilis S	0	+/-	+/-	+/-	+/+

[1] Ap = ampicillin trihydrate, Cm = chloramphenicol, Sm = streptomycin sulfate, Su = 2-sulfanimidopyrimidine, Tc = tetracycline hydrochloride

[2] Strain designations: S = sewage isolate, H = hospital isolate

[3] 0 = no initial resistance, + = resistance, - = no resistance

[4] Pre-curing resistance listed in numerator, post-curing in denominator

[5] Recombinant is listed first, donor from which the recombinant received resistance is listed second

Table 6. Resistance curing by 10^{-5} M ethidium bromide

Organism	Resistance				
	Ap[1]	Cm	Sm	Su	Tc
S. enteritidis H[2]	0[3]	0	+/+[4]	+/+	0
P. mirabilis H	0	0	+/+	+/+	+/+
P. mirabilis S	0	+/+	+/+	+/+	+/+
E. coli H1	+/+	0	+/-	+/-	0
E. coli H2	+/-	0	+/+	+/-	0
E. coli S1	+/+	0	+/-	+/+	0
E. coli S2	+/+	+/-	0	+/+	0
S. sonnei S - *E. coli* H1[5]	+/-	0	+/+	+/-	0
S. sonnei S - *E. coli* H2	+/-	0	+/-	+/-	0
S. sonnei S - *E. coli* S1	+/-	0	+/+	+/-	0
S. sonnei S - *E. coli* S2	+/-	+/-	0	+/-	0
S. sonnei S - *P. mirabilis* H	0	0	+/-	+/+	+/-
S. sonnei S - *P. mirabilis* S	0	+/-	+/-	+/+	+/-
E. coli H3 - *S. enteritidis* H	0	0	+/-	+/-	0
E. coli H3 - *P. mirabilis* H	0	0	+/-	+/-	+/+
E. coli H3 - *P. mirabilis* S	0	+/-	+/+	+/+	+/+

[1] Ap = ampicillin trihydrate, Cm = chloramphenicol, Sm = streptomycin sulfate, Su = 2-sulfanilamidopyrimidine, Tc = tetracycline hydrochloride

[2] Strain designations: S = sewage isolate, H = hospital isolate

[3] 0 = no initial resistance, + = resistance, - = no resistance

[4] Pre-curing resistance listed in numerator, post-curing in denominator

[5] Recombinant is listed first, donor from which the recombinant received resistance is listed second

Table 7. Summary of curable resistance

Organism	Resistance				
	Ap[1]	Cm	Sm	Su	Tc
S. enteritidis H[2]	0[3]	0	+/+[4]	+/+	0
P. mirabilis H	0	0	+/-	+/+	+/+
P. mirabilis S	0	+/-	+/+	+/+	+/+
E. coli H1	+/-	0	+/+	+/+	0
E. coli H2	+/-	0	+/+	+/+	0
E. coli S1	+/+	0	+/-	+/+	0
E. coli S2	+/+	+/-	0	+/+	0
S. sonnei S - *E. coli* H1[5]	+/-	0	+/-	+/-	0
S. sonnei S - *E. coli* H2	+/-	0	+/-	+/-	0
S. sonnei S - *E. coli* S1	+/-	0	+/-	+/-	0
S. sonnei S - *E. coli* S2	+/-	+/-	0	+/-	0
S. sonnei S - *P. mirabilis* H	0	0	+/-	+/+	+/-
S. sonnei S - *P. mirabilis* S	0	+/-	+/-	+/+	+/+
E. coli H3 - *S. enteritidis* H	0	0	+/-	+/-	0
E. coli H3 - *P. mirabilis* H	0	0	+/-	+/-	+/+
E. coli H3 - *P. mirabilis* S	0	+/-	+/-	+/-	+/+

[1]Ap = ampicillin trihydrate, Cm = chloramphenicol, Sm = streptomycin sulfate, Su = 2-sulfanilamidopyrimidine, Tc = tetracycline hydrochloride

[2]Strain designations: S = sewage isolate, H = hospital isolate

[3]0 = no initial resistance, + = resistance, - = no resistance

[4]Pre-curing resistance listed in numerator, post-curing in denominator

[5]Recombinant is listed first, donor from which the recombinant received resistance is listed second

However, 85.7% of the resistance received from the donor species was eliminated from the recipient organisms by curing.

Transduction and transformation studies were conducted as previously described. However, no recombinants were demonstrated among the recipient cells placed in the clarifiers as controls.

Discussion

Transferable antibiotic resistance has been demonstrated in bacterial communities from a variety of environments. This ubiquitous nature of R plasmid containing bacteria, especially those from humans and domestic animal sources (71), has helped public health officials become aware of the hazards of indiscriminant use of antibiotics for nonmedical purposes.

The purpose of this study was to demonstrate antibiotic resistance transfer in a wastewater treatment plant. Specifically, the objectives were to:

i. demonstrate resistance transfer as it occurred between treatment plant and hospital isolates in the wastewater treatment plant,

ii. determine the frequency of resistance transfer, and

iii. demonstrate the plasmid (Rplasmid) nature of all transferable resistance observed.

High levels of R plasmid-containing bacteria have been identified in normal sewage effluent by Sturtevant and Feary (177) and by Sturtevant et al. (178). These bacteria comprised 0.5-1.0% of the total coliform population observed at the sewage outfall. Hospital effluent has been shown to contain even higher numbers of R plasmid-containing bacteria. Grabow and Prozesky (66) and Fontaine and Hoadley (57) demonstrated that 26% and 56%, respectively, of the coliform bacteria in hospital effluent possessed transferable resistance. The relatively high percentages of bacteria with transferable resistance found before and after treatment eluded the high levels of resistant bacteria in the wastewater treatment plant (67,70,110). Thus, because

resistant organsims have been identified in both hospital and normal sewage effluent, bacterial strains used for the transfer studies were isolated from primary effluent (S) and from patients during diagnostic analysis at a nearby hospital.

High level antibiotic resistance was demonstrated among isolates from both sewage effluent and from the hospital environment (Table 1). Fontaine and Hoadley (57) and Linton et al. (111) also showed similar resistance levels among isolates from hospital and sewage effluent. Generally, resistance to antimicrobial agents was seen to reach its highest levels in the presence of the antibiotics (153). However, because little difference was evident when comparing inhibitory concentrations among isolates from the two environments, the resistance characteristics may have provided an ecological advantage in both hospital and sewage environments because of the variety of resistance factors seen among most R plasmids.

Multiple drug resistance, as seen among sewage and hospital isolates, was typical of organisms found in an environment possessing more than one type of antibiotic (153). The prevalence of multiple resistance among sewage isolates (Table 1) agreed with the findings of Cooke (36), who showed a high incidence of multiply-resistant coliforms in sewage and in sea water containing the sewage effluent. Grabow et al. (67) and Grabow and Prozesky (66) have shown progressively higher levels of multiple resistance as wastewater treatment proceeds toward the plant outfall and also in the water beyond the outfall. The basis of this observation has yet to be fully demonstrated but R plasmid properties (e.g. heavy metal resistance), not ecologically

related to (but covalently bonded with) antibiotic resistance may provide the selective pressure necessary for production and maintenance of the multiple resistance (71).

The production and maintenance of the multiple resistance has also been shown among the flora of persons having no known exposure to antibiotics. Datta (42) and Widh and Skold (198) demonstrated similar resistance patterns (i.e., antibiotic types and resistance concentrations) among the flora of hospitalized and non-hospitalized individuals. This ubiquitous demonstration of resistance, even among bacterial populations not exposed to antibiotics, leads one to believe that a reservoir exists for resistance.

Transfer kinetics have thus far been studied only in defined, in vitro systems. Harada and Mitsuhashi (76) has studied the transfer kinetics for E. coli-Shigella systems. They revealed temperature and pH to be the primary abiotic factors controlling in vitro transfer. For these parameters, they found temperature ranges of 25 to 40°C and pH ranges of 5.0 to 9.0 to support R plasmid transfer. Harada and Mitsuhashi further demonstrated 37°C and pH 7.5 to be optimum for resistance transfer. Transfer frequencies reported by Harada and Mitsuhashi (76) and ranged from 10^{-2} to 10^{-4}. Thosevalues are in general agreement with the values obtained in this study for in vitro transfer frequencies observed in NB and SS (Table 2). Both media (NB and SS) were complex and undefined. Therefore, an unbiased comparison of the two was possble only if the assumption was made that neither possessed constituents that significantly inhibited cell growth and reproduction. The difference between NB and SS, with respect to growth potential was illustrated in the growth curve determinations

(Appendices 4-9). The curves for SS approximate log death. The fact that the transfer rate in NB was significantly greater ($P < .03$) than in SS reflects this obvious chemical difference in media type.

Resistance transfer frequencies for in situ primary and secondary clarifiers are listed in Table 2. Transfer frequencies for the primary clarifier ($\bar{x} = 4.3 \times 10^{-5}$) were significantly less ($P < .1$) than the rate of resistance transfer observed in secondary clarification ($\bar{x} = 7.5 \times 10^{-5}$). From studies conducted with their membrane-filter chambers, McFeters and Stuart (114) revealed almost free, large molecular weight (approximately 5×10^5) chemical exchange from outside to inside. This demonstration, together with the wide acceptance for use of the chamber for in situ studies in aquatic envionments led to the use of this diffusion chamber for the wastewater treatment plant in situ studies. THerefore, the chemical differences demonstrated in the clarifiers directly affected the bacterial interactions within the membrane-diffusion chamber. Turbulence would not have a direct effect on the internal environment of the chamber, but physical water movements outside the chamber may have resulted in chamber displacement in the water column, which would simulate the external turbulence.

Physical-chemical differences for the two clarifying tank environments are listed in Appendix 2. Beyond obvious pH differences (primary clarifier, $\bar{x}_{pH} = 8.4$; secondary clarifier, $\bar{x}_{pH} = 7.5$) differences in suspended solids (S.S.) and biochemical oxygen demand (B.O.D.) revealed significant differences in the chemical constituents of the two clarifiers. These chemical differences may explain the variation in transfer frequency observed between the matings in the two clarification tanks. Primary clarification turbulance was visibly greater than that seen in the

secondary clarifier. Thus, physical movement of the transfer chambers in primary clarification may have reuslted in breakage of the fragile conjugal pili (66,67).

When comparing individual organisms for the intergeneric matings, in situ, the stability of E. coli H3 showed higher transfer frequencies in both primary and secondary clarification (primary, $\bar{x} = 3.7 \times 10^{-5}$; secondary, $\bar{x} = 12.6 \times 10^{-5}$), than did Shigella sonnei S (primary, $\bar{x} = 3.6 \times 10^{-5}$; secondary, $\bar{x} = 10.6 \times 10^{-5}$). These observations revealed E. coli as a relatively stable interspecific donor and recipient of antibiotice resistance in situ. For this reason, E. coli may function as a relatively nonvirulent pathogenic mediator or antibiotic resistance transfer among strains with greater virulence (e.g., Salmonella enteritidis). In vivo studies have repeatedly shown resistance transfer to occur between E. coli and S. enteritidis ser. Typhimurium (170,171). However, the present study was the first to describe simulated in situ transfer with potentially pathogenic organisms.

The simulation of in situ transfer revealed a decrease in transfer frequency as also described by Lacey (103) when comparing artificial to natural conditions. THerefore, he insisted in vivo transfer could not be inferred from in vitro tests. This observation also led him to believe transfer amond enteric pathogens did not occur to any significance in vivo. However, Smith (171), Anderson (12) and Kayser et al. (97) have shown the significance of in vivo resistance transfer among hospitalized patients. Their studies revealed that although the rate of transfer is significantly decreased by natural environmental conditions, lowered transfer frequencies were especially significant when the transfer took place in the proper, selective environment (i.e., high antibiotic concentration).

Lowered transfer frequencies have also been demonstrated for in situ simulation studies comparing transfer rates in a river water medium with rates seen in a nutrient broth. Grabow et al. (69) showed a decline of 10^{-3} from river water frequencies to those calculated for an undefined nutrient broth. Although no explanation was offered for this drastic decline in transfer rate, Geldrich (63) has postulated the presence of complex bactericidal activity associated with river water. Other factors which may have led to the decline in resistance transfer are: pilus receptor site interference, pilus injury, as well as bacterial predation and parasitism. These interactions may also be present in wastewater. Consequently, bacteria exposed to this variety of factors would be killed, or lose transfer capabilities resulting in decreased transferable resistance associated with the bacterial community. This decline in transferable resistance would ultimately result in a decreased transfer frequency in the wastewater treatment plant.

To establish the plasmid nature of resistance and transferable resistance in both donor and recombinant strains, attempts were made to eliminate the extrachromosomal genetic elements from all strains that demonstrated resistance to the five antibiotics. Due to their extrachromosomal nature (32), plasmids are eliminated from the host bacterium following exposure of that bacterium to sub-lethal concentrations of intercalcating dyes (e.g., acridine orange, ethidium bromide) (153,152,151,190). Also, because genetic material associated with pilus synthesis is found on the R plasmid, detergents (e.g., dodecyl sodium sulfate) that eliminate bacterial cells possessing these special pili also eliminate the R plasmids (156).

Using these chemical treatments, resistance was eliminated from 69.4% of all strains (Table 7). The removal of resistance by exposure

to curing agents was especially effective for recombinant strains. The 85.7% curing rate seen among recombinant strains corresponds well with the 80-90% curing rates observed for E. coli and Shigella species by Mitsuhashi et al. (118) and by Salisbury et al. (156). Therefore, the high-level of resistance shown, (Table 1), high resistance curing rates observed among donor and recombinant organisms (Table 7) and the transmissible nature of the resistance (Table 2) suggested that the resistance observed in this study was, infact, R plasmid-mediated (71).

This study revealed the in situ transfer of antibiotic resistance among isolated bacteria from the wastewater treatment plant and from a nearby hospital. THe in situ transfer frequencies, although lower than those typically observed in vitro, demonstrated a high incidence of R plasmid transfer among bacteria within the wastewater treatment plant. Due to the diversity of the microbial community entering the wastewater treatment plant, R plasmids originating from non-pathogenic bacteria may be conjugally-transferred to pathogens. The selective pressure for the incidence of multiple-resistance as the wastewater treatment process proceeds (68,71), may result in the release of a multiply-resistant, pathogenic bacterial population into the aquatic environment. Presence of these resistant pathogens would severely limit the use of such water for recreational or commercial purposes. THe incidence of a water-borne, Shigella-outbreak was recently reported immediately below a wastewater treatment plant (25,150). Such occurrences of water-borne disease by multiply-resistant bacteria should lead to evaluation of not only the efficiency of the treatment plant and the use of water below the outfall of a wastewater treatment plant, but also an

evaluation of the use of antibiotics for non-medical purposes. Obviously, the antibiotic concentrations within the treatment plant was not the selective factor facilitating the maintenance and transfer of antibiotic resistance. However, the selective environment which allowed the evolution and maintenance of a transferable plasmid with multiple antimicrobial resistance (e.g., heavy metals, ultra-violet radiation, antibiotics) must be regulated to minimize such genetic recombinations.

CONCLUSIONS

In this study, antibiotic resistance transfer has been demonstrated in vitro, and in situ. The significance of differences in transfer frequencies commonly seen for these environments is dependent on the selective nature of the environment. Because R plasmids code for resistance to a variety of agents (e.g., antibiotics, heavy metals, ultraviolet radiation), the selective nature of a specific environment is not necessarily high antibiotic concentration. However, acknowledging the widespread use of antibiotics for commercial and medical purposes, an extremely efficient selective environment is maintained for the evolution and maintenance of multiply-resistant bacteria in animal husbandries, in commercial fisheries and in hospitals. With the large antibiotic dosages required for treatment of large animals or large numbers of animals, fishery ponds and human beings, antibiotic overflow may occur, resulting in selective pressure for other environments to maintain a resistant flora. Other such environments include wastewater treatment plants. For this reason, a need has arisen for careful regulation and control of the use of antibiotics for non-medical, non-infectious purposes.

From this simulation study, several conclusions can be drawn with regard to transfer of antibiotic resistance in wastewater treatment plants:

i. High levels of antibiotic resistance occur among the flora of both hospitalized patients and wastewater treatment plants.

ii. Antibiotic resistance transfer does occur in situ in the primary and secondary clarifiers of a wastewater treatment plant.

iii. A significant difference in transfer frequency exists between primary to secondary clarification environments. The difference appears to be due to the variation in physical-chemical factors for the two clarifiers.

iv. Transfer frequencies vary among the donor-recipient pairings that occur. However, E. coli was shown to be a relatively stable interspecific donor and recipient of antibiotic resistance.

v. Transformation and bacteriophage transduction did not significantly add to the amount of antibiotic resistance transferred with the wastewater treatment plant.

vi. The environment present in the wastewater treatment plant was adequately represented by the environment within the modified Stuart-McFeters chamber. Although bacterial contaminants and other larger species were kept fromentering the diffusion chamber, physical-chemical alterations in the treatment plant directly or indirectly affected the mating medium within the diffusion chamber.

vii. Antibiotic resistance present among bacteria isolated from wastewater treatment and hospitalized individuals was transferred to sensitive strains from the same environments at rates that may be significant in receiving water beyond the sewage outfall.

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Appendix 1. Monthly average physical-chemical constituent for the wastewater treatment plant, 1979

Month	Physical-chemical measurement				
	pH	S.S.[1]	B.O.D.[2]	D.O.[3]	S.V.I.[4]
Jan	8.6/7.4	95/16	438/16	5.4	122
Feb	8.5/7.4[5]	89/11	481/15	4.5	125
Mar	8.4/7.3	102/11	429/22	4.5	141
Apr	8.6/7.4	92/9	447/15	4.3	169
May	8.2/7.4	83/8	363/10	4.5	163
Jun	8.5/7.4	89/22	400/24	4.2	179
Jul	8.4/7.4	74/17	359/16	4.1	143
Aug	8.6/7.4	80/5	384/9	3.8	297
Sep	8.3/7.4	80/6	374/15	3.8	319
Oct	8.5/7.5	81/15	453/25	3.3	302
Nov	8.3/7.4	89/15	404/18	4.1	355
Dec	8.4/7.4	303/11	423/22	4.6	143

[1] S.S. = total suspended solids (mg/L)

[2] B.O.D. = biochemical oxygen demand (5 day, 20°C)

[3] D.O. = dissolved oxygen (mg/L) for final effluent

[4] S.V.I. = sludge volume index (ml/L) for final effluent

[5] For all fractions listed, numerator indicates value for primary clarifier, denominator indicates value for secondary

Appendix 2. Wastewater treatment plant physical-chemical constituents, 2-9 October 1979

Date	Physical-chemical measurements					
	T[1]	pH	S.S.[2]	B.O.D.[3]	D.O.[4]	S.V.I.[5]
2nd	13.3	8.6/7.4	69/7[6]	420/18	3.9	346
3rd	13.3	7.9/7.4	67/7	400/11	3.8	305
4th	7.8	8.9/7.5	67/6	413/13	3.8	313
5th	12.2	9.1/7.5	63/6	366/16	3.5	303
6th	9.4	7.9/7.5	66/7	427/9	3.6	291
7th	8.9	7.8/7.4	82/6	400/8	3.6	277
8th	10.6	8.0/7.5	77/5	387/17	3.6	283
9th	8.9	9.0/7.4	82/5	420/37	3.5	306

[1]T = water temperature in ^{o}C as measured for the primary effluent

[2]S.S. = total suspended solids (mg/L)

[3]B.O.D. = biochemical oxygen demand (5 day, $20^{o}C$)

[4]D.O. = dissolved oxygen (mg/L) for final effluent

[5]S.V.I. = sludge volume index (ml/L) for final effluent

[6]For all fractions listed, numerator indicates value for primary clarifier, denominator indicates value for secondary

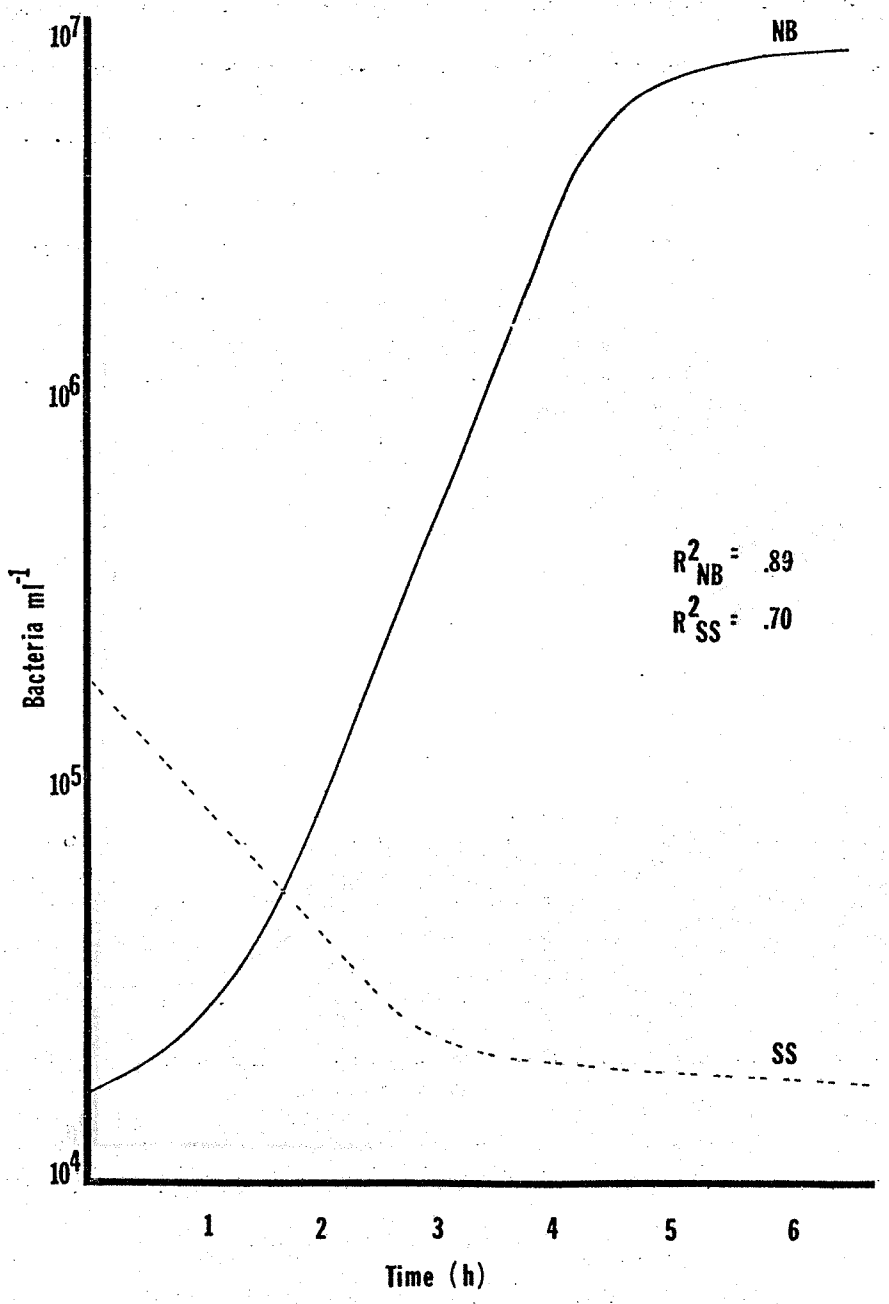
Appendix 3. Standard Biochemical Set[1] results for sewage and hospital isolates

Test[2]	S. enteritidis H[3]	P. mirabilis H	P. mirabilis S	E. coli H1	E. coli H2	E. coli H3	E. coli S1	E. coli S2	S. sonnei S
H_2S	+	+	+	-	-	-	-	-	-
Urease	-	+	+	-	-	-	-	-	-
Indole	-	-	-	+	+	+	+	+	-
MR	+	+	+	+	+	+	+	+	+
VP	-	-	-	-	-	-	-	-	-
Citrate	+	-	-	-	-	-	-	-	-
KCN	-	+	+	-	-	-	-	-	-
Motility	+	+	+	+	+	+	+	+	-
LD	+	-	-	+	+	+	+	+	-
AD	+	-	-	-	-	-	+	-	-
OD	+	+	+	+	-	+	+	-	+
PD	-	+	+	-	-	-	-	-	-

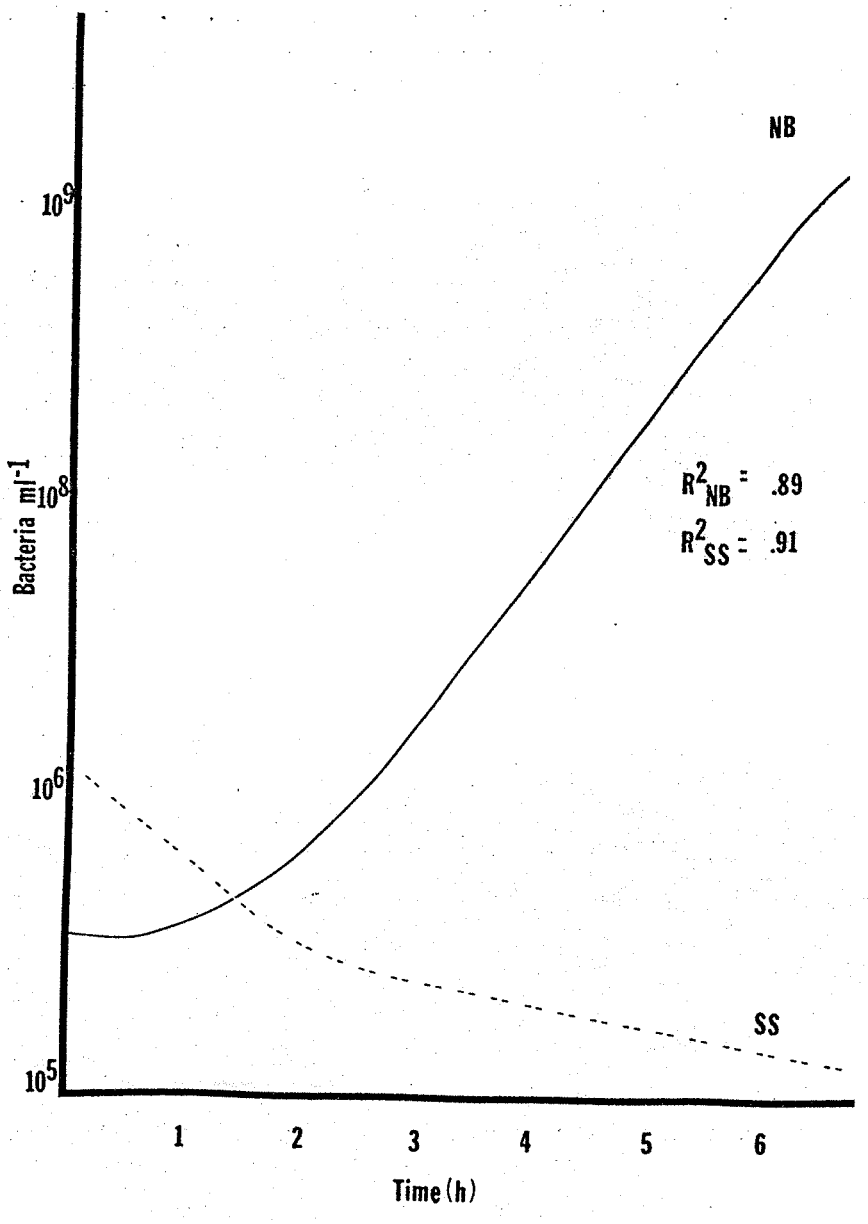
[1]Edwards and Ewing, 1973 (52)

[2]Tests are designated: MR = methyl red, VP = Voges-Proskauer LD = lysine decarboxylase, AD = arginine dihydrolase, OD = ornithine decarboxylase, PD = phenylalanine decarboxylase

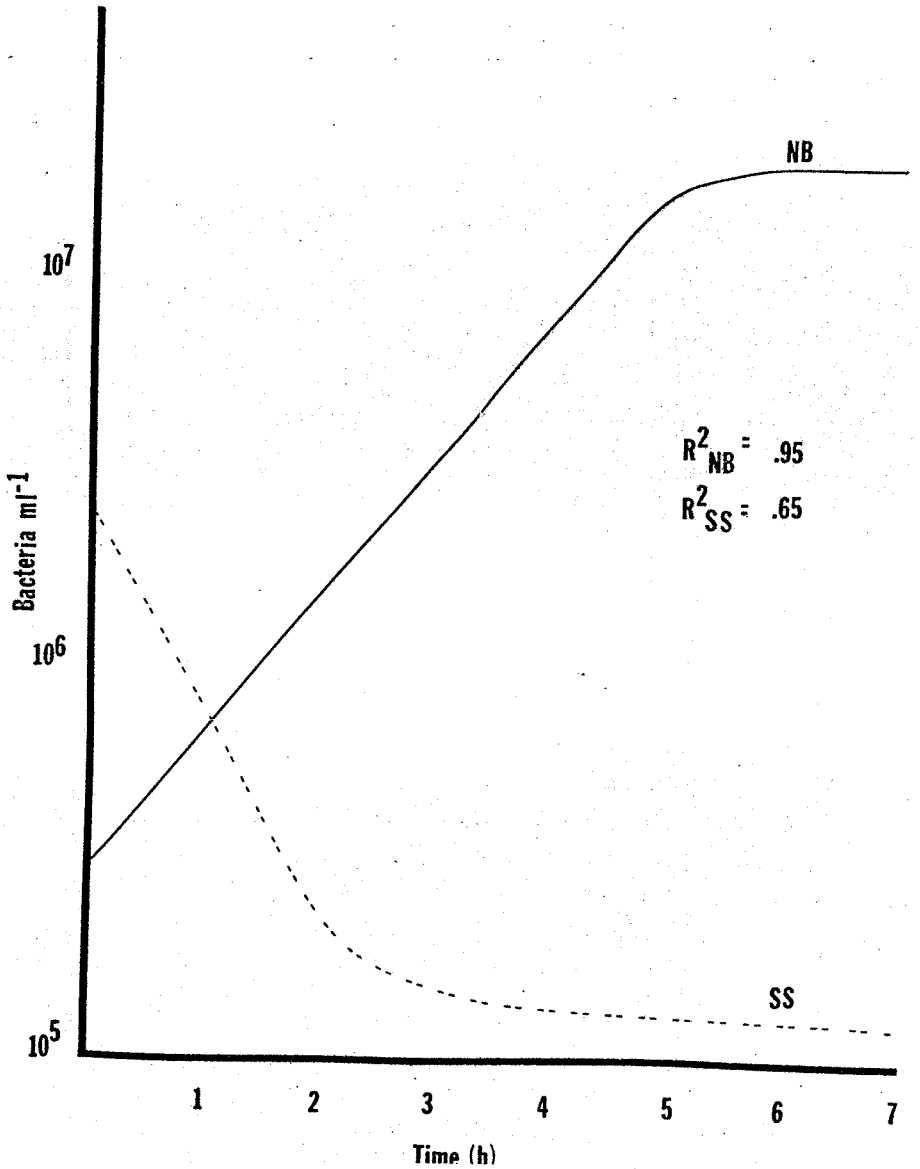
[3]Strain designation: H = hospital isolate, S = sewage isolate



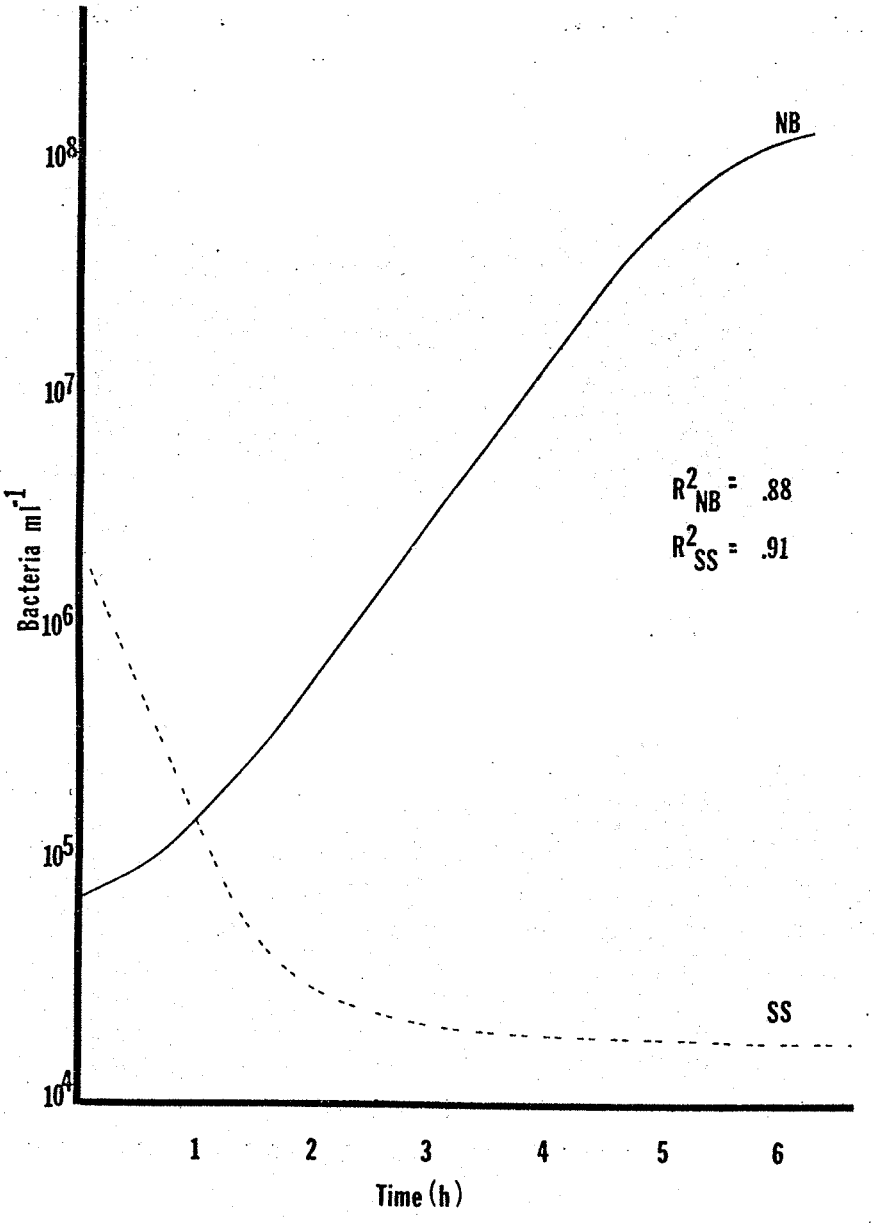
Appendix 4. Growth of Salmonella enteritidis H in Nutrient broth (NB) and sterile sewage (SS). Multiple correlation coefficients for the plotted lines are listed for each medium (R^2_{NB} and R^2_{SS}).



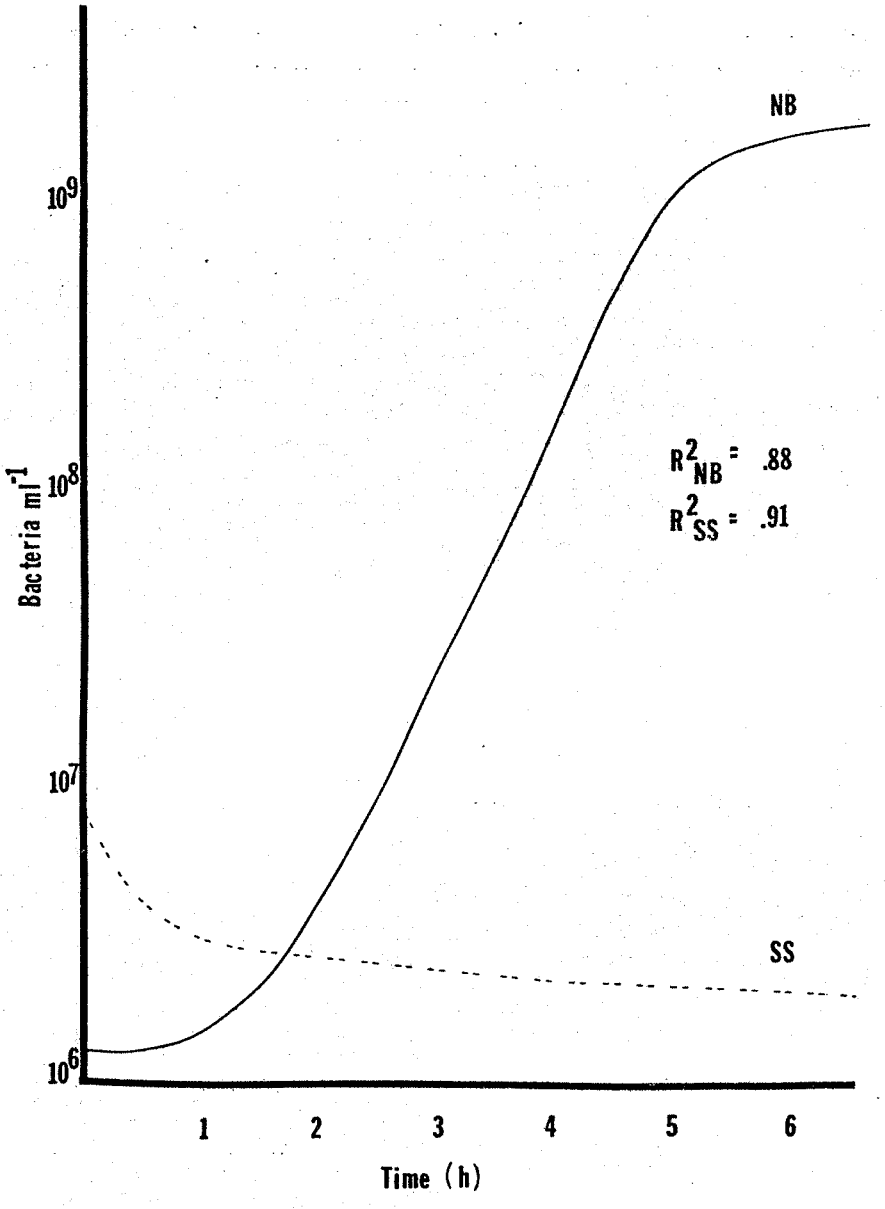
Appendix 5. Growth of Proteus mirabilis H in Nutrient broth (NB) and sterile sewage (SS). Multiple correlation coefficients for the plotted lines are listed for each medium (R^2_{NB} and R^2_{SS}).



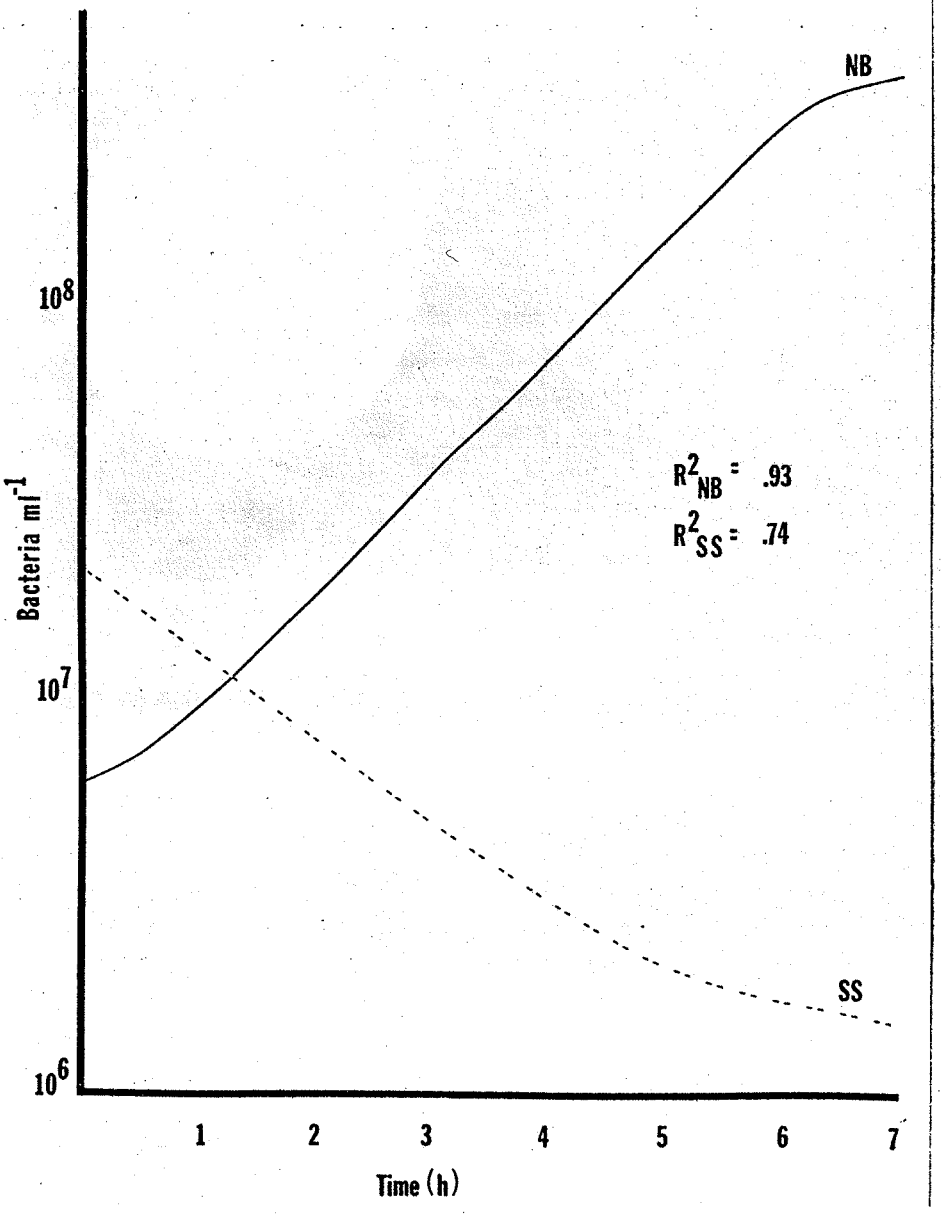
Appendix 6. Growth of Proteus mirabilis S in Nutrient broth (NB) and sterile sewage (SS). Multiple correlation coefficients for the plotted lines are listed for each medium (R^2_{NB} and R^2_{SS}).



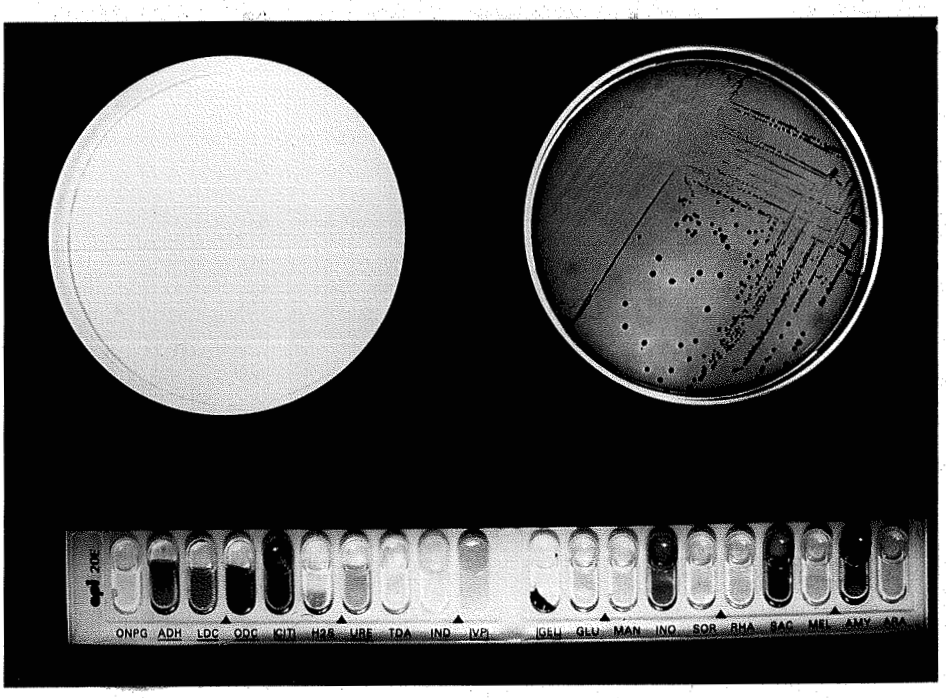
Appendix 7. Growth of Escherichia coli H1 in Nutrient broth (NB) and sterile sewage (SS). Multiple correlation coefficients for the plotted lines are listed for each medium (R^2_{NB} and R^2_{SS}).



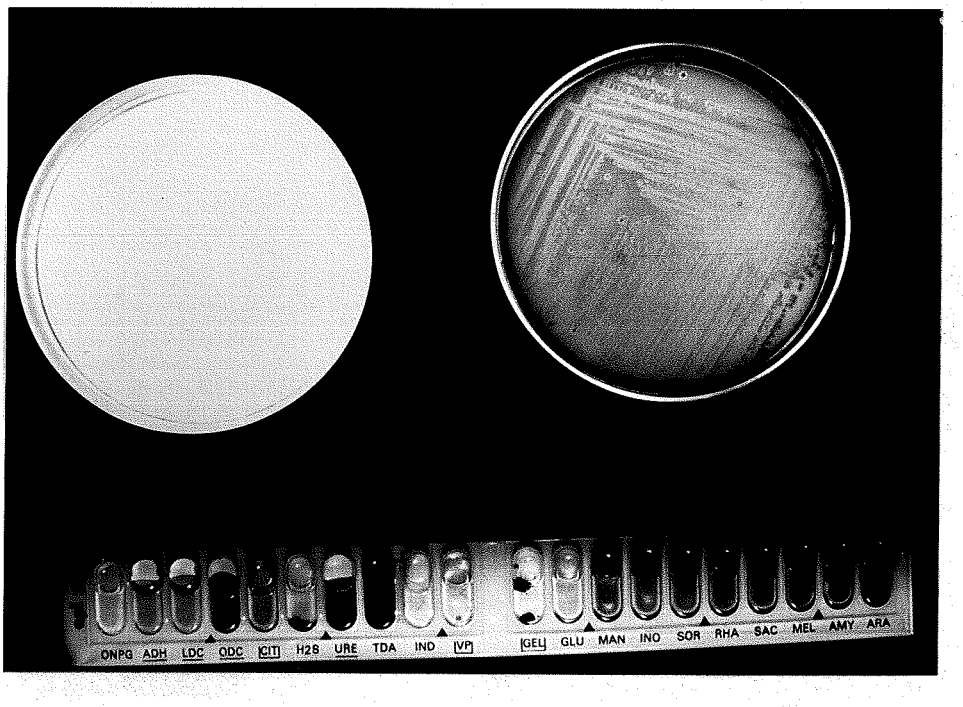
Appendix 8. Growth of Escherichia coli S2 in Nutrient broth (NB) and sterile sewage (SS). Multiple correlation coefficients for the plotted lines are listed for each medium (R^2_{NB} and R^2_{SS}).



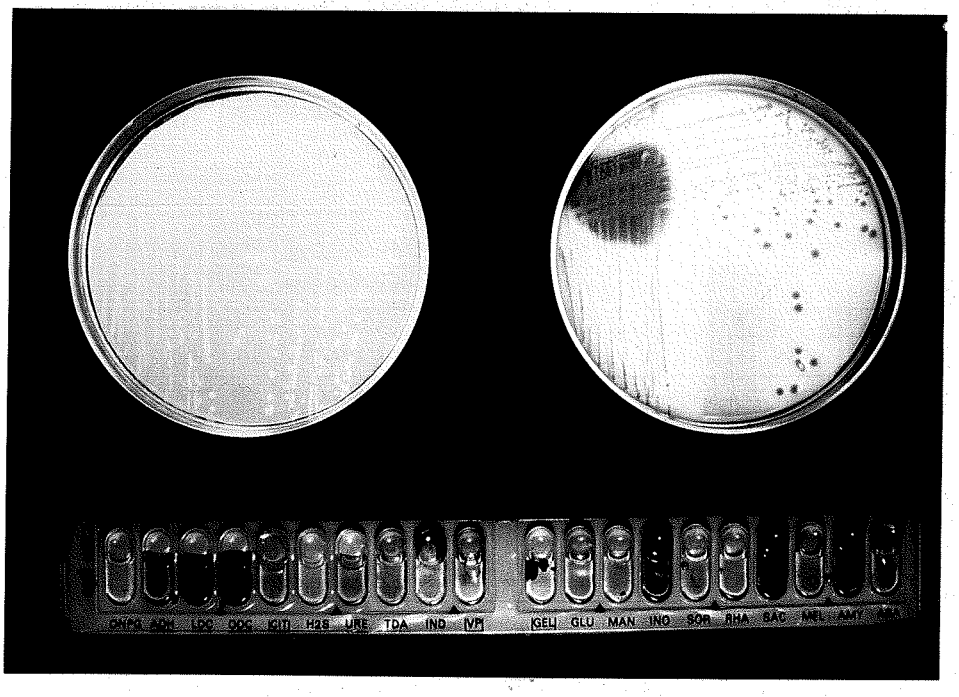
Appendix 9. Growth of Shigella sonnei S in Nutrient broth (NB) and sterile sewage (SS). Multiple correlation coefficients for plotted plotted lines are listed for each medium (R^2_{NB} and R^2_{SS}).



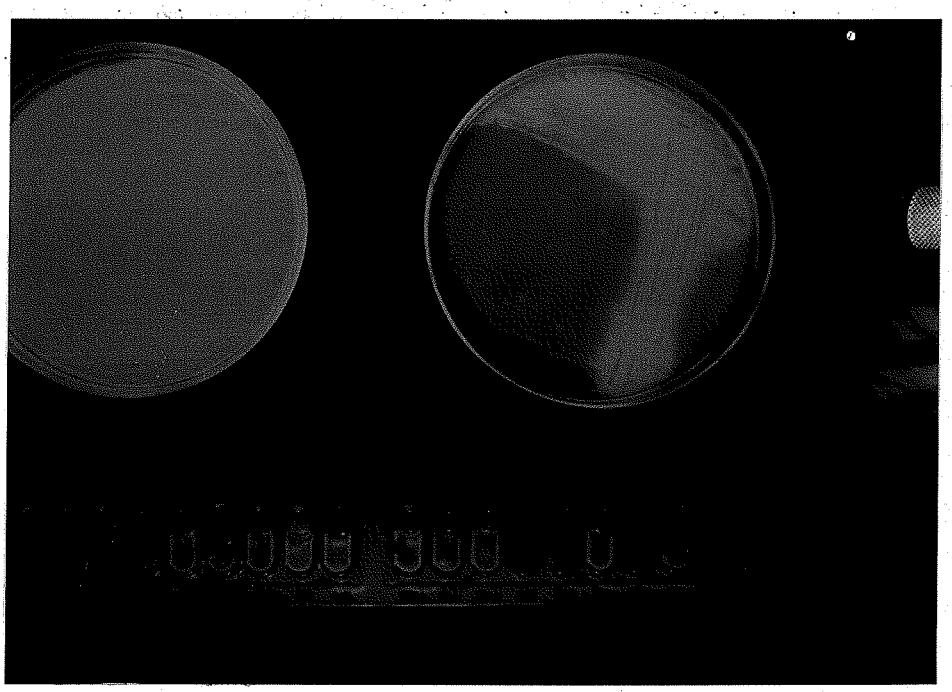
Appendix 10. Nutrient and XLD agar plate colonial morphology and API 20E reactions of Salmonella enteritidis H following 24-h incubation at 35°C.



Appendix 11. Nutrient and XLD agar plate colonial morphology and API 20E reactions of Proteus mirabilis H following 24-h incubation at 35°C.



Appendix 12. Nutrient and XLD agar plate colonial morphology and API 20E reactions of Escherichia coli H1 following 24-h incubation at 35°C.



Appendix 13. Nutrient and XLD agar plate colonial morphology and API 20E reactions of Shigella sonnei S following 24-h incubation at 35°C.