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R-Plasmid Transfer in a Wastewater Treatment Plant

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Enteric bacteria have been examined for their ability to transfer antibiotic resistance in a wastewater treatment plant. Resistant *Salmonella enteritidis*, *Proteus mirabilis*, and *Escherichia coli* were isolated from clinical specimens and primary sewage effluent. Resistance to ampicillin, chloramphenicol, streptomycin, sulfadiazine, and tetracycline was demonstrated by spread plate and tube dilution techniques. Plasmid mediation of resistance was shown by ethidium bromide curing, agarose gel electrophoresis, and direct cell transfer. Each donor was mated with susceptible *E. coli* and *Shigella sonnei*. Mating pairs (and recipient controls) were suspended in unchlorinated primary effluent that had been filtered and autoclaved. Suspensions were added to membrane diffusion chambers which were then placed in the primary and secondary setting tanks of the wastewater treatment plant. Resistant recombinants were detected by replica plating nutrient agar master plates onto xylose lysine desoxycholate agar plates that contained per milliliter of medium 10 μg of ampicillin, 30 μg of chloramphenicol, 10 μg of streptomycin, 100 μg of sulfadiazine, or 30 μg of tetracycline. Mean transfer frequencies for laboratory matings were 2.1×10^{-3}. In situ matings for primary and secondary settling resulted in frequencies of 4.9×10^{-5} and 7.5×10^{-5}, respectively. These values suggest that a significant level of resistance transfer occurs in wastewater treatment plants in the absence of antibiotics as selective agents.

With the advent of the age of antimicrobial agents, medical and veterinary science gained very promising means for treating bacterial infections in humans and in other animal species. However, almost from the beginning, the seemingly hypermutable bacteria demonstrated an ability to resist bacteriostatic and bactericidal effects of antimicrobial agents.

The most common genetic instrument for resistance among bacteria is, of course, the R plasmid (6, 20). Acquisition of these plasmids occurs via all three types of recombination (transformation, viral transduction, and conjugation), although conjugation appears to be the most common method for in vivo transfer (6, 20). Resistance transfer among gram-negative rods has posed a significant threat in human nosocomial infections (13, 24) and in other animal infections where antimicrobial prophylaxis has provided the selective pressure necessary to maintain R plasmids in the etiological agent(s) (22, 31). The relative ease with which bacteria become resistant to currently used antimicrobial agents has been and continues to be of concern to public health officials (16, 20, 32).

Because of the high incidence of resistance among nosocomial bacteria, considerable research has been conducted on in vivo R-plasmid transfer. These studies have demonstrated that transfer does occur, and at relatively high rates, in wounds (21, 24) and in the gastrointestinal (3, 35), urinary, and respiratory tracts (13) of warm-blooded animals, including humans.

Bacteria isolated from a variety of aquatic and terrestrial habitats have also demonstrated resistance to antimicrobial agents. Generally, bacteria with the greatest levels of resistance have been isolated from environments with the greatest potential for significant contamination by antimicrobial agents, e.g., hospitals and hospital sewage effluent (9, 12, 17), commercial fisheries (40), and abattoirs (15, 31). However, resistant bacteria have also been isolated from apparently nonselective environments, including plants (38), estuaries (28), deep ocean water and sediment (19, 34), and drinking water (4).

Isolation of R-plasmid-bearing bacteria from natural environmental sources has led to much speculation about the possibility of in situ resistance transfer, the frequency with which it occurs, and its effect on public health. Recently,

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research on this aspect of antibiotic-resistant bacteria has been associated with wastewater contamination of water resources (7, 12, 15, 16, 18, 26, 32). However, transfer of resistance has not been demonstrated among environmental isolates within the confines of a wastewater treatment plant; this, therefore, is the subject of this paper.

MATERIALS AND METHODS

Site description. In situ resistance transfer studies were conducted in the primary and secondary clarifiers of the La Crosse Wastewater Treatment Plant, La Crosse, Wis. The plant treats approximately 6.4×10^6 liters of sewage per day by the activated sludge method. Daily averages of pH, suspended solids removal, biochemical oxygen demand removal, dissolved oxygen concentration, and sludge volume index as measured by the treatment plant during the study period (2 to 9 October 1979) are listed in Table 1. Sludge analysis by the plant revealed a mercury level of 3.44 mg of Hg per kg (dry weight) of sludge collected on 2 to 9 October 1979.

Isolation techniques. Bacterial isolates were obtained from primary sewage effluent from the La Crosse Municipal Wastewater Treatment Plant and from the La Crosse Lutheran Hospital bacteriology laboratory. Bacteria were isolated from primary effluent by standard enrichment techniques (2). Tetrathionate and gram-negative broths (Difco Laboratories) were used as the selective enrichment media for *Salmonella* and *Shigella* isolations, respectively. Approximately 0.5 liter of primary effluent was filtered through a 0.45-μm membrane filter (HCWG-047-00; Millipore Corp.). The filter was then placed into 10 ml of broth; tetrathionate broth tubes were incubated at 41.5°C for 24 h, and gram-negative broth tubes were incubated at 35°C for 18 to 24 h. Tubes demonstrating turbidity were streaked onto xylose lysine desoxycholate (XLD) agar (Difco) plates for isolation and preliminary identification. Isolated colonies revealing colonial characteristics typical of either genus were identified by the method of Edwards and Ewing (10). Biochemically presumptive *Salmonella* spp. and *Shigella* spp. were serologically confirmed and grouped (*Salmonella* and *Shigella* Typing Serum Kits, BBL Microbiology Systems). *Proteus* spp. and *Escherichia coli* isolations were facilitated by serial 1:10 dilutions of wastewater samples in 0.85% (wt/vol) NaCl. Countable plates (30 to 300 colonies) were obtained by spread plating 0.1-ml samples onto XLD agar. Typical colonies were selected and identified as previously described (10). These methods provided isolations of *E. coli* (two strains), *Proteus mirabilis* (one strain), and *Shigella sonnei* group D (one strain) from primary effluent. Clinical isolates of *Salmonella enteritidis* group B (one strain), *P. mirabilis* (one strain), and *E. coli* (three isolates) were identified by hospital personnel using the API 20E system (Analytab Products). These identifications were confirmed in our laboratory (10). Stock cultures of hospital (H) and sewage (S) isolates were maintained at room temperature (20°C) in motility test medium (BBL) 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, chloramphenicol, streptomycin sulfate, 2-sulfanilamidopyrimidine (sulfadiazine), and tetracycline hydrochloride; and for resistance to $HgCl_2$. Sources of the biochemicals were as follows: ampicillin and sulfadiazine, United States Biochemical Corp.; chloramphenicol, streptomycin, and tetracycline, Calbiochem-Behring Corp.; $HgCl_2$, Fisher Scientific Co. Fresh 125-ml solutions of each compound (1 to 4 mg/ml) were prepared weekly, filter sterilized, and refrigerated (4°C). Appropriate quantities of chloramphenicol, streptomycin, tetracycline, and $HgCl_2$ were suspended in distilled water. Ampicillin was suspended in 10% (vol/vol) methanol. Sulfadiazine was suspended in 0.1 N NaOH.

Resistance was demonstrated by tube dilution in nutrient broth (Difco). Bacteria were grown in 10 ml of nutrient broth for 6 h, at which time 1 ml of 1:10 dilutions (0.85% NaCl) of each culture (200 to 500 cells per ml) was transferred to 9 ml of nutrient broth containing antimicrobial agents in concentrations of 10, 30, 50, 100, 500, 1,000, 1,500, and 2,000 μg/ml. Tubes were incubated at 35°C for 18 h, after which growth was determined by absorbance at 620 nm (Spectronic 20, Bausch & Lomb, Inc.). The minimum

TABLE 1. Physical-chemical data for the La Crosse Municipal Wastewater Treatment Plant, 2 to 9 October 1979

Date	Temp (°C)[a]	pH[b]	Suspended solids (mg/liter)[b]	Biochemical oxygen demand (mg/liter)[b]	Dissolved oxygen (mg/liter)[c]	Sludge vol index (ml/liter)[c]
2	13.3	8.6/7.4	69/7	420/18	3.9	346
3	13.3	7.9/7.4	67/7	400/11	3.8	305
4	7.8	8.9/7.5	67/6	413/13	3.8	313
5	12.2	9.1/7.5	63/6	366/16	3.5	303
6	9.4	7.9/7.5	66/7	427/9	3.6	291
7	8.9	7.8/7.4	82/6	400/8	3.6	277
8	10.6	8.0/7.5	77/5	387/17	3.6	283
9	8.9	9.0/7.4	82/5	420/37	3.5	306

[a] Measured for the primary effluent.
[b] First number shows value for primary clarifier; second number shows value for secondary clarifier.
[c] Measured for final effluent.

inhibitory concentration (MIC) was considered to be the lowest concentration of antibiotic which produced no significant absorbance. None of the bacteria exhibited sensitivity to methanol as evidenced by negligible absorbance differences between the nutrient broth controls and cultures grown in nutrient broth that contained 10% methanol (concentration of methanol in tubes containing 2,000 μg of ampicillin per ml).

Growth curve determinations. Bacterial growth curves were determined for each species growing in nutrient broth and in sewage effluent. The sewage effluent was prefiltered through type AP prefilters (AP25-047-00, Millipore), passed through 0.45-μm membrane filters, autoclaved, and designated sterile sewage. Each medium (nutrient broth or sterile sewage) was seeded with a 24-h nutrient broth culture that had been visually standardized against McFarland Standard no. 5 (1.5×10^9 bacteria per ml, Difco) with 0.85% NaCl as the diluent. Cultures were then enumerated at 0.5-h intervals by absorbance at 620 nm and by spread plating 0.1 ml of appropriate dilutions onto nutrient agar (Difco) plates. Cell counts were plotted as a function of absorbance to obtain standard curves for resistance transfer studies.

Transfer studies. In vitro and in situ resistance transfer studies were conducted for each mating pair. In vitro studies were conducted in both nutrient broth and sterile sewage in the laboratory; in situ studies were conducted only with sterile sewage at the treatment plant. Transfer frequencies (see Table 3) were calculated as the numbers of resistant recipient bacteria arising per resistant donor, given as the means of duplicate experiments.

In vitro transfer was studied with 20 ml of medium at 20°C. Approximately 2.9×10^6 each of resistant (donor) and susceptible (recipient) late log phase bacteria of different species were inoculated into the growth medium (nutrient broth or sterile sewage). Transfer studies were conducted for 3 h; at 1-h intervals, 0.1-ml samples were removed and diluted in 0.85% (wt/vol) NaCl. Appropriate dilutions were then spread plated onto nutrient agar plates. Countable plates were then replica plated onto XLD agar plates containing antibiotics which served to distinguish species composition and resistance to antimicrobial agents. Concentrations of antimicrobial agents, in micrograms per milliliter, were: ampicillin, 10; chloramphenicol, 30; streptomycin, 10; sulfadiazine, 100; and tetracycline, 30 (12). Colonies identified in this manner were confirmed with API 20E strips on at least two colonies per XLD plate.

In situ transfer studies were completed in primary and secondary clarifiers with modified membrane filter diffusion chambers (11) as the containment device. Modifications included increasing the thickness of the central spacer (from 6.5 to 12 mm) to provide increased volume within the chamber (from 20 to 30 ml) and the addition of plastic screening (mesh, 0.635 cm) over the surface of the membranes. Each chamber was fitted with 0.4-μm polycarbonate membranes (no. 11507, Nuclepore Corp.) and then sterilized with ethylene oxide. The chambers were loaded with 20 ml of sterile sewage and allowed to equilibrate with the aquatic environment in the treatment plant for approximately 0.5 h. Equilibrated chambers were then inoculated with sterile sewage suspensions of donor and recipient bacteria to a final concentration of 2.9×10^6 cells per ml each. In situ studies were conducted for 3 h. At 1-h intervals, 0.1-ml samples were removed and diluted in 0.85% (wt/vol) NaCl. The resistance of each conjugant to antimicrobial agents and its bacterial identification were demonstrated as described previously.

Transduction and transformation. The possible role of transformation or viral transduction in mediating antibiotic resistance transfer in the wastewater treatment plant was investigated by placing membrane diffusion chambers inoculated with antibiotic-susceptible (recipient) bacteria, i.e., *Shigella sonnei* S and *E. coli* H3, into the clarifying tanks. Chambers were inoculated with 4×10^{12} bacteria per ml of equilibrated sterile sewage; this large inoculum permitted the detection of recombinant rates with a minimum frequency of approximately 10^{-11} per recipient. No steps were taken to distinguish between transformation and transduction.

Plasmid detection and curing. Plasmids were detected by a modification of the rapid lysis technique of Kado and Liu (23). Our modification consisted of using polypropylene tubes (12 by 75 mm) with snap caps, heating the cells in lysing solution at 65°C for 20 min, and extracting each twice with two volumes of phenol-chloroform. The aqueous layer (450 μl) was then withdrawn into a 1.5-ml microcentrifuge tube and adjusted to 0.3 M Na acetate by adding 50 μl of 3 M Na acetate. The plasmid DNA was precipitated by a 15-min exposure to two volumes of cold (−20°C) 95% ethanol. Plasmid DNA was then sedimented by a 5-min centrifugation in a microcentrifuge, reprecipitated with cold 70% ethanol, and recentrifuged. The tubes were allowed to air dry inverted over clean lab tissue. The DNA was then reconstituted in 100 μl of the Kado and Liu dye. Gels were photographed on a model C-62 transilluminator (Ultra-Violet Products, Inc.) with type 55 film (Polaroid Corp.) exposed through Wratten no. 9, 2B, and 23A gelatin filters (Eastman Kodak Co.) simultaneously. The molecular weights of donor and recipient plasmids were mathematically determined by the equation $y = bx^m$, where y is the molecular weight, b is the y intercept, x is the relative mobility, and m is the slope. Relative mobility-molecular weight data points obtained from standard plasmid-containing bacteria (27, 28) were included with each plasmid detection experiment.

Resistance curing experiments were conducted on late logarithmic phase donor and recombinant bacterial populations. The curing agents used were 25 and 50 μg of acridine orange (AO; Eastman Kodak Co.) per ml (30), 1% sodium dodecyl sulfate (SDS; Eastman) (1), and 10^{-5} M ethidium bromide (EB; Calbiochem-Behring). These compounds were added separately to nutrient agar plates. Curing was accomplished by spread plating 0.1 ml of a 1:10 dilution of 6-h nutrient broth cultures onto the plate. Cultures were then replica plated onto unaltered (no agent present) nutrient agar plates, which served as the master templates for replica plating onto XLD agar. Antibiotics to which the organisms had previously shown resistance were incorporated in these XLD plates to determine curing patterns. Cultures were considered cured if there was at least a 100-fold decrease in the number of colonies growing on XLD-antibiotic plates as compared with the nutrient agar master plates.

RESULTS

Antibiotic resistance. Resistances of *Salmonella enteritidis* H, *P. mirabilis* H, *P. mirabilis* S, *E. coli* H1, *E. coli* H2, *E. coli* H3, *E. coli* S1, *E. coli* S2, and *Shigella sonnei* S to ampicillin, chloramphenicol, streptomycin, sulfadiazine, and tetracycline are listed in Table 2. Two of the nine strains (*E. coli* H3 and *Shigella sonnei* S) had no demonstrable resistance to the five antibiotics and were used as recipients for in vitro and in situ transfer studies. Maximum resistance was seen toward sulfadiazine ($\bar{x}_{MIC}$ = 2,000 μg/ml), followed by tetracycline ($\bar{x}_{MIC}$ = 1,500 μg/ml), chloramphenicol ($\bar{x}_{MIC}$ = 1,500 μg/ml), streptomycin ($\bar{x}_{MIC}$ = 566 μg/ml), and ampicillin ($\bar{x}_{MIC}$ = 100 μg/ml). Furthermore, all resistant bacteria demonstrated multiple drug resistance, and those showing resistance to three of the five antibiotics were the most common (71.4%); resistance to two or four antibiotics was not as prevalent (14.3%). High-level chloramphenicol resistance was demonstrable only for sewage isolates; streptomycin resistance was more prevalent among hospital isolates. Otherwise, antibiotic resistance to the chemical agents was evenly distributed among strains from both environments.

Transfer frequencies. Results of resistance transfer to *E. coli* H3 and *Shigella sonnei* S are listed in Table 3. *Shigella sonnei* S was mated with all seven isolates showing resistance; however, to facilitate cultural identification of donor and recipient strains, *E. coli* H3 was not crossed with resistant *E. coli* (strains H1, H2, S1, and S2). All resistance acquisition occurred by conjugation since no recombinants were demonstrated among the susceptible recipient strains placed in the clarifiers as transduction-transformation controls. In vitro transfer of antibiotic resistance occurred more often ($\bar{x} = 2.1 \times 10^{-3}$ per donor) than did in situ transfer ($\bar{x} = 5.9 \times 10^{-5}$ per donor), and mean transfer frequencies from nutrient broth studies ($\bar{x} = 2.1 \times 10^{-3}$ per donor) were similar to those in sterile sewage ($\bar{x} = 2.2 \times 10^{-3}$ per donor). However, close examination revealed that in vitro sterile sewage resistance transfer did not occur for all matings, and statistical analysis (Student *t* test) confirmed this observation ($P < 0.03$).

For in situ matings, transfer frequencies were greater in the secondary clarifier ($\bar{x} = 7.5 \times 10^{-5}$) than in the primary clarifier ($\bar{x} = 4.9 \times 10^{-5}$) ($P < 0.1$). There was also a difference in transfer frequency between the two recipients. In vitro and in situ frequencies revealed *E. coli* H3 recombinant rates ($\bar{x} = 2.7 \times 10^{-3}$) to be slightly higher than those for *Shigella 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. coli* donors mated with the sensitive *Shigella sonnei* recipient (Table 3).

Plasmid detection. Plasmid bands exhibiting similar electrophoretic mobilities were observed in agarose gels of both the recombinants and their respective donors. *E. coli* H3 and *Shigella sonnei* S did not contain detectable plasmids. Figure 1 shows a typical gel. Clearly detectable bands document a plasmid basis for resistance transfer from *Salmonella enteritidis* H to *E. coli* H3. Estimation of the molecular weights of the three plasmid bands observed in *Salmonella enteritidis* H (Fig. 1, well C) and recombinant *E. coli* H3 (Fig. 1, well D) resulted in values of 42, 20, and 18 megadaltons (Mdal). Logarithmic plots of molecular weight as a function of relative mobility for the eight plasmids in *E. coli* V517 (27) and the four in *E. coli* H10407 (28) always produced a straight line and gave in this case (Fig. 1) a correlation coefficient of −0.971 ($P < 0.01$). Calculation of the molecular weights by the equation $y = bx^m$ yielded values of 31, 21, and 18 Mdal for *Salmonella enteritidis* H and recombinant *E. coli* H3. Plasmid maintenance in both donors and recombinants has been stable, with slightly less than 50% of the cultures losing their plasmids over 2 years of storage in motility test medium without antibiotics.

Curable resistance. Antibiotic resistance elimi-

TABLE 2. Resistance of donor and recipient bacteria to various antimicrobial agents

Isolate	MIC (μg/ml)					
	Ampicillin	Chloramphenicol	Streptomycin	Sulfadiazine	Tetracycline	$HgCl_2$
Salmonella enteritidis H	<10	<10	100	2,000	<10	100
P. mirabilis H	<10	<10	100	2,000	1,500	<10
P. mirabilis S	<10	1,500	100	2,000	1,500	<10
E. coli H1	100	<10	1,500	2,000	<10	<10
E. coli H2	100	<10	1,500	2,000	<10	<10
E. coli H3	<10	<10	<10	<10	<10	<10
E. coli S1	100	<10	100	2,000	<10	1,000
E. coli S2	100	1,500	<10	2,000	<10	100
Shigella sonnei S	<10	<10	<10	<10	<10	<10

TABLE 3. Frequency of resistance marker transfer between donor and recipient bacteria

Organisms (donor × recipient)	Antibiotic	R-plasmid transfer frequencies ($\times 10^{-4}$) in[a]:			
		Nutrient broth	Sterile sewage	Primary clarifier	Secondary clarifier
S. enteritidis H × *E. coli* H3	Streptomycin	52	29	0.8	2.7
	Sulfadiazine	38	35	0.8	2.0
S. enteritidis H × *S. sonnei* S	Streptomycin	22	23	0.2	0.3
	Sulfadiazine	27	15	0.2	1.0
P. mirabilis H × *E. coli* H3	Streptomycin	23	27	0.4	0.4
	Sulfadiazine	27	21	0.4	1.0
	Tetracycline	13	0	0.0	0.3
P. mirabilis H × *S. sonnei* S	Streptomycin	10	0	0.0	0.9
	Sulfadiazine	10	0	0.7	1.2
	Tetracycline	7	0	0.0	0.0
P. mirabilis S × *E. coli* H3	Chloramphenicol	5	0	0.0	0.0
	Streptomycin	38	14	0.4	1.2
	Sulfadiazine	27	9	0.4	1.2
	Tetracycline	20	0	0.4	0.0
P. mirabilis S × *S. sonnei* S	Chloramphenicol	0	0	0.0	0.0
	Streptomycin	3	0	0.0	1.0
	Sulfadiazine	5	0	0.4	1.7
	Tetracycline	5	24	0.4	1.3
E. coli H1 × *S. sonnei* S	Ampicillin	10	19	0.5	0.2
	Streptomycin	20	14	0.3	0.2
	Sulfadiazine	27	10	0.4	0.2
E. coli H2 × *S. sonnei* S	Ampicillin	18	10	0.5	0.1
	Streptomycin	28	14	0.4	0.1
	Sulfadiazine	7	7	0.5	0.1
E. coli S2 × *S. sonnei* S	Ampicillin	60	45	0.7	0.5
	Streptomycin	22	19	0.4	0.4
	Sulfadiazine	30	23	0.5	0.3
E. coli S2 × *S. sonnei* S	Ampicillin	23	43	0.3	0.5
	Chloramphenicol	15	17	0.4	0.2
	Sulfadiazine	5	36	0.3	0.5

[a] Means of duplicate determinations.

nation with AO, SDS, and EB was conducted on resistant donor and resistant recombinant organisms (Table 4). Resistance was eliminated with both 25 and 50 μg of AO per ml. Generally, the curing effect of these two concentrations was equal; the absence of detectable plasmids in recombinant *E. coli* H3 mated to *Salmonella enteritidis* H and then cured with 25 and 50 μg of AO per ml is shown in Fig. 1, wells F and G. However, only 50 μg of AO per ml eliminated streptomycin and tetracycline resistance from the recombinant *Shigella sonnei* S that had been mated with *E. coli* H1 and *P. mirabilis* H, respectively. Resistance elimination by SDS cured 57% of resistance from donors growing in its presence; *P. mirabilis* isolates failed to grow on SDS-nutrient agar. A similar elimination rate (46%) was observed among resistant recombinant organisms. When 10^{-5} M EB was used, much of the resistance exhibited by the recombinants was eliminated (71.4%), whereas a smaller percentage (28.6%) was cured among donor organisms. Overall, among donor organisms, 57% of the demonstrable resistance was eliminated by exposure to the curing agents, compared with 85.7% among recombinant populations.

DISCUSSION

High-level resistance to five antibiotics was demonstrated among seven isolates from sewage effluent and from hospitalized patients. The MICs (Table 2) generally agreed with the levels seen by Fontaine and Hoadley (12) and Linton et al. (26) for hospital and domestic sewage effluent

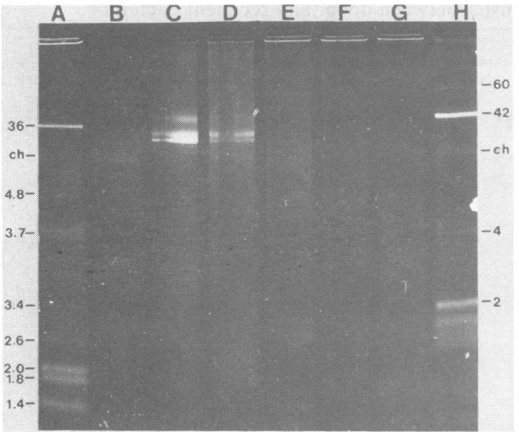

FIG. 1. Agarose gel electrophoretic analysis of selected donor, recipient, recombinant, and reference strains. (A) Reference *E. coli* V517 (27) with plasmids of 1.4, 1.8, 2.0, 2.6, 3.4, 3.7, 4.8, and 36 Mdal; (B) recipient *E. coli* H3; (C) donor *Salmonella enteritidis* H with bands at 18, 21, and 31 Mdal; (D) recombinant *E. coli* H3 × *Salmonella enteritidis* H with bands at 18, 21, and 31 Mdal; (E) recipient *Shigella sonnei* S; (F) recombinant shown in track D after curing with 25 μg of AO per ml; (G) same as track F, but cured with 50 μg of AO per ml; (H) *E. coli* H10407 (28) with plasmids of 2, 4, 42, and 60 Mdal. ch, Chromosome.

isolates. Resistance correlates with the level of antibiotic exposure (3, 26), and in general, 2 to 5% of the coliforms in sewage contain R plasmids, whereas over 50% of the coliforms in hospital wastewater may carry them (12, 17, 26, 37). Surprisingly, little difference was evident between MICs for our isolates from these two very different environments. Multiple resistance was prevalent (Table 2), in agreement with the findings of Cooke (7), who showed high numbers of multiple-resistant coliforms in sewage and in seawater polluted with sewage effluent. The resistance pattern observed in *Salmonella enteritidis* (Table 2) was the second most common pattern detected by Tanaka et al. (39) in a study of 1,980 *Salmonella* strains. Grabow et al. (16) and Grabow and Prozesky (17) have shown a tendency for progressively larger numbers of multiple-resistant organisms to occur as sewage treatment proceeds toward the plant outfall and the receiving water. Kushner has observed that exposure to chlorine in sewage plants and elsewhere leads to an increased frequency of antibiotic resistance in coliform bacteria (D. J. Kushner, personal communication). The basis of these observations has yet to be fully explained, but it may involve R plasmids that code for resistance to a variety of abiotic factors (e.g., heavy metals, UV radiation) not ecologically related to, but transferred with, antibiotic resistance. These other factors may provide the selective advantage necessary for maintaining multiple resistance. The treatment plant used in this study contained detectable amounts of mercury in the sludge, and three of our isolates exhibited resistance to $HgCl_2$. However, transfer and curing of mercury resistance were not examined.

The use of membrane filter chambers allowed for simulation and detection of in situ resistance transfer between environmental and clinical iso-

TABLE 4. Summary of curable resistance

Organism	Resistance to[a]:				
	Ampicillin	Chloramphenicol	Streptomycin	Sulfadiazine	Tetracycline
Donor					
S. enteritidis H	0	0	RRRR	RRRR	0
P. mirabilis H	0	0	SS−R	RR−R	RR−R
P. mirabilis S	0	SS−R	RR−R	RR−R	RR−R
E. coli H1	SRSR	0	SSSS	SSSS	0
E. coli H2	SRSS	0	SSSR	SSSS	0
E. coli S1	RRRR	0	SSSS	RSRR	0
E. coli S2	RRRR	SSSS	0	RSRR	0
Recombinant (donor × recipient)					
E. coli H1 × *S. sonnei* S	SSRS	0	RSRR	SSSS	0
E. coli H2 × *S. sonnei* S	RRSS	0	SSSS	RRSS	0
E. coli S1 × *S. sonnei* S	SSSS	0	RSRR	RSSS	0
E. coli S2 × *S. sonnei* S	RRSS	RRRS	0	RRSS	0
P. mirabilis H × *S. sonnei* S	0	0	SSRS	RRRR	RSRS
P. mirabilis S × *S. sonnei* S	0	SSSS	SSRS	RRRR	SSRS
S. enteritidis H × *E. coli* H3	0	0	SSRS	SSRS	0
P. mirabilis H × *E. coli* H3	0	0	SSSS	RSRS	RRRR
P. mirabilis S × *E. coli* H3	0	SSSS	RSSR	RRSR	RRRR

[a] The four letters in each entry show resistance after exposure to 25 μg of AO per ml, 50 μg of AO per ml, 1% SDS, and 10^{-5} M EB, in that order. Symbols: 0, No initial resistance; R, resistant; S, susceptible; −, no growth.

lates. In addition, the use of natural isolates, rather than highly mutated fragile laboratory strains (e.g., *E. coli* K-12 strains) no doubt facilitated transfer. Heretofore, in situ resistance has been demonstrated in but a few select environments. Smith (35) was able to demonstrate that R-containing *E. coli* transfers resistance to susceptible *Salmonella enteritidis* serotype typhimurium recipients in the rumen of sheep. Anderson et al. (3) demonstrated that R plasmids were transferred between *E. coli* strains contained within the gastrointestinal tracts of three healthy human volunteers. However, transfer occurred only after the volunteers had been placed on a regimen of oral antibiotic, which acted as a selective pressure. Hummel et al. (21) succeeded in transferring resistance to four aminoglycoside antibiotics from a resistant *Alcaligenes* sp. to a susceptible *Pseudomonas aeruginosa*. The transfer was accomplished by inoculating susceptible *P. aeruginosa* into 10% third-degree burns on germfree mice that had been previously monocontaminated with an oral inoculation of resistant *Alcaligenes* sp. The authors suggested R-plasmid transfer as the probable mechanism but did not experimentally demonstrate the presence of such plasmids. Baross et al. (5) demonstrated transduction of the agarase characteristic to *Vibrio parahaemolyticus* contained in aquarium-held oysters. Grabow et al. (18) were able to detect resistance transfer from resistant to sensitive *E. coli* contained in dialysis bags that were placed in a South African river. The frequency of transfer was low (only 30% of the donors transferred the trait in situ), and actual plasmid transfer was not demonstrated. More recently, Morrison et al. (29) demonstrated in situ transduction of streptomycin resistance to susceptible *P. aeruginosa*. The bacteria were mixed with generalized transducing phage F116 in a cylindrical membrane diffusion chamber, and transduction frequencies ranging from 10^{-5} to 10^{-2} were observed. Stewart and Koditschek (36) reported transfer of drug resistance (but not of plasmids) between laboratory strains of *E. coli* in sterile New York Bight sediment that was contained in cylindrical glass vessels and covered with sterile seawater.

Transfer kinetics have thus far been studied in depth only in defined in vitro systems. Harada and Mitsuhashi (20), studying the transfer kinetics of *Escherichia-Shigella* systems, showed temperature and pH to be the primary abiotic factors controlling in vitro transfer. They found that temperature ranges of 25 to 45°C (37°C optimum) and pH ranges of 5.0 to 9.0 (7.5 optimum) supported plasmid transfer. Singleton and Anson (33) examined the transfer of plasmid R1*drd-19* between laboratory strains of *E. coli* contained in nutrient broth. They observed maximal transfer at 37°C, with transfer frequencies decreasing directly with temperature; no transfer was detected at 15°C. Our in vitro studies were conducted at 20°C, a temperature observed by Singleton and Anson (33) and by Grabow et al. (18) to support low, but significant, rates of transfer. Interestingly, and in contradiction with in vitro studies on nutrient media, Grabow et al. (18) found 20°C to be more conducive than 37°C to R-plasmid transfer between *E. coli* strains contained in river water in the laboratory. Transfer frequencies reported by Harada and Mitsuhashi (20) and by Shaw and Cabelli (32) were 10^{-2} to 10^{-4} and 1 to 10^{-6}, respectively. These in vitro transfer frequencies were also in general agreement with in vitro transfer frequencies obtained in this study (Table 3).

In situ transfer in the primary and secondary clarifiers differed in frequency (Table 3) and may have been influenced by physical and chemical differences between the two clarifying tank environments (Table 1). Beyond obvious pH differences, differences in suspended solids and in biochemical oxygen demand support the conclusion that the chemical environment (inorganic and organic) in secondary clarification was more conducive to interbacterial transfer of antibiotic resistance than was the chemical environment in primary clarification. Further, turbulence in the primary clarifier was visibly greater than turbulence in the secondary clarifier; this may have led to more frequent breakage of fragile conjugal pili (1, 6, 18) and could be the most important variable between the two systems.

Transfer frequencies observed in the primary and secondary clarifiers were lower than those observed in vitro (Table 3), and this may have been due to temperatures below optimum. Water temperatures averaged 10.6°C (Table 1), well below those previously reported to be supportive of conjugation (18, 20, 33). Lowered transfer frequencies were also observed by Grabow et al. (18) in complex media. Although Grabow et al. (18) offered no explanation for this decline in transfer frequency, Geldreich (14) has postulated complex antibacterial activity associated with river water. Other factors which may have led to the decline in resistance transfer observed by Grabow et al. (18) and in the present study are pilus receptor site interference, pilus injury, formation of cell aggregates, bacterial predation and parasitism, and absence of antibiotic pressure. It is doubtful that superinfection inhibition (6, 20) was involved since neither of the recipients contained detectable plasmids (Fig. 1).

In comparisons of intergeneric in situ matings, recipient *E. coli* H3 showed higher transfer frequencies in both primary and secondary clarification than did *Shigella sonnei* S. These observations revealed *E. coli* to be a relatively stable

recipient of antibiotic resistance in situ and agreed with the findings of Sizemore and Colwell (34). In a laboratory study of plasmid spread through model populations of *E. coli*, Cullem et al. (8) demonstrated that plasmid retransfer by recipients may be a very important factor in the movement of plasmids through and between populations. It is clear, then, that strains of *E. coli* may serve as efficient mediators of transferable resistance among bacteria with greater virulence, e.g., *Salmonella enteritidis*, both in vitro and in situ.

To establish the plasmid nature of resistance and resistance transfer in both donor and recombinant strains, attempts were made to eliminate the extrachromosomal genetic elements from all strains. Because they are extrachromosomal (6), plasmids are eliminated from host bacteria after exposure to sublethal concentrations of intercalating dyes (e.g., AO, EB) (6, 30). Also, certain detergents (e.g., SDS) tend to lyse piliated bacteria selectively, thereby eliminating all plasmids carried by those cells (1). These chemical treatments eliminated resistance from 69.4% of all strains. Curing was especially effective for recombinant strains; the 85.7% curing rate seen among recombinant strains corresponds well with the 80 to 90% rates observed for *E. coli* by Adachi et al. (1) and by Salisbury et al. (30). Therefore, the high level of resistance (Table 2), high curing frequencies (Table 4), and the transmissible nature of resistance (Table 3) suggest that the resistance observed in this study was R-plasmid mediated. This hypothesis was confirmed by the detection in agarose gel electrophoresis of similarly migrating plasmid bands in donors and recombinants, but not in recipients and cured strains (Fig. 1).

In conclusion, the demonstration of simulated in situ R-plasmid transfer between bacteria contained in a sewage treatment plant is an important step toward understanding the impact of wastes on autochthonous aquatic microorganisms. Although the frequencies of transfer were low (10^{-5}) when compared with those in controlled laboratory matings (10^{-3}), bacteria in a sewage treatment plant are certainly present in high enough numbers and in close enough proximity to one another to effect significant transfer and retransfer of R plasmids and of other transferable extrachromosomal genetic elements, including chimeric plasmids (25).

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