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In Situ Studies with Membrane Diffusion Chambers of Antibiotic Resistance Transfer in *Escherichia coli*

MICHAEL R. ALTHERR† AND KENNETH L. KASWECK*

Department of Biological Sciences, Florida Institute of Technology, Melbourne, Florida 32901

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Coliform bacteria were isolated from raw sewage and sewage effluent-receiving waters and tested for their antibiotic susceptibility patterns and their ability to transfer antibiotic resistance to *Escherichia coli* K-12 C600. An environmental isolate of *E. coli* (MA527) capable of transferring antibiotic resistance to C600 was mated, both in vitro and in situ, with an antibiotic-sensitive *E. coli* environmental isolate (MA728). In situ matings were conducted in modified membrane diffusion chambers, in the degritter tank at the Grant Street (Melbourne, Fla.) sewage treatment facility, and in the sewage effluent-receiving waters in Melbourne, Fla. The transfer frequencies in situ were 3.2×10^{-5} to 1.0×10^{-6}, compared with 1.6×10^{-4} to 4.4×10^{-5} observed in vitro. Transfer was shown to occur in raw sewage but was not detected in the effluent-receiving waters. The presence of a 60-megadalton plasmid species in both donor and transconjugants, but not in the recipients, provided physical evidence for the transfer of antibiotic resistance in situ.

The transfer of antibiotic resistance in bacteria has been the subject of much research and numerous reviews (2, 4, 8, 19, 27, 28, 36). Antibiotic-resistant bacteria have been isolated from raw sewage (7, 13, 21, 26), sewage effluent-receiving waters (16, 34), fresh and marine recreational waters (10, 19, 30, 31), and marine shellfish (6), coastal sediments (17), and soil (5). The transfer of resistance has been observed between environmental isolates containing R plasmids, designated R^+, and various recipients in vitro (15, 29), but little work has been conducted in the field (17). Some investigators have attempted to recreate in situ conditions and have shown that antibiotic resistance transfer occurs in these simulated environments (13, 33). The ubiquity of bacteria possessing transferable antibiotic resistance presents obvious health hazards and dictates the need for an experimental system that will provide a greater understanding of the in situ behavior of R plasmids.

Membrane diffusion chambers have proven to be versatile tools for studying the effects of environmental stress on bacteria. They have been used to study the survival and viability of coliforms in a variety of aquatic systems (3, 9, 14, 24), the survival and viability of *Aeromonas hydrophila* in a thermally altered lake (12), and lactose variability in *Escherichia coli* after exposure to thermally stressed reactor effluent waters (20). Modified membrane diffusion chambers are inexpensive, simple to construct, easy to work with, and suitable for conducting field studies on the in situ behavior of bacteria possessing R plasmids (11).

MATERIALS AND METHODS

Strains and culture conditions. The strains used in this study are listed in Table 1. All of the strains were routinely maintained on nutrient agar (Difco Laboratories, Detroit, Mich.). To prevent the spontaneous loss of the R plasmid from the donor strain, MA527 was maintained on nutrient agar supplemented with 15 μg of tetracycline per ml.

Study sites. The study sites (Fig. 1) were within the boundaries of the city of Melbourne, Fla. The sites used to determine coliform counts were a pipe entering the sewage treatment plant from a residential area (site A), the sewage treatment facility degritter tank (site B), the final effluent just before release (site C), and a small eddy in Crane Creek adjacent to the effluent release point (site D). In situ matings were conducted in the degritter tank at the sewage treatment facility and in Melbourne Harbor approximately 500 m downstream of the effluent release point (site E).

Total coliform counts, isolation, and characterization of potential donors. Total coliform counts were determined at the four sites described above by the membrane filtration (1) to determine the ability of the sites to support coliform populations.

Antibiotic-resistant bacteria were obtained by direct selection at 37°C on plates of MacConkey agar (Difco) supplemented with the following antibiotics at a final concentration of 15 μg/ml: chloramphenicol, streptomycin, and tetracycline (Sigma Chemical Co., St.

† Present address: Department of Genetics, University of California, Davis, CA 95616.

TABLE 1. Strains used in transfer studies

Strain[a]	Source	Properties[b]
C600	R. Sparks, Connecticut Agricultural Station	Nal^r, Lac$^-$, F$^-$, plasmidless
MA527	Residential sewage, Melbourne, Fla.	Sm^r, Tc^r, Tra$^+$, ADH$^+$, LDC$^+$, ODC$^+$, AMY$^+$, contains 60-megadalton plasmid[c]
MA728	Crane Creek, Melbourne, Fla.	Nal^r, Tra$^-$, ADH$^-$, LDC$^-$, AMY$^-$

[a] Strain C600 is an *E. coli* K-12 derivative. MA527 was determined to be *E. coli* by using the API 20E system and was rated as an "excellent identification" by the API 20E Analytical Profile Index. MA728 was determined to be *E. coli* by the API 20E system and was rated as a "very good identification" by the Analytical Profile Index.

[b] Nal, Nalidixic acid; Sm, streptomycin; Tc, tetracycline; Lac, lactose; F, fertility; Tra, transferable resistance; r, resistant; ADH, LDC, ODC, and AMY refer to the following API 20E biochemical tests: arginine dehydrolase, lysine decarboxylase, ornithine decarboxylase, and amygdalin fermentation, respectively.

[c] Designated pKK1.

Louis, Mo.). Lactose-fermenting colonies were streak purified by repeated transfer to nutrient agar (Difco), Levine eosin methylene blue agar (Difco), and finally to nutrient agar supplemented with the antibiotic (15 μg/ml) to which the strain was resistant. Strain identification was accomplished with the API 20E system (Analytab Products, Plainview, N.Y.). Susceptibility testing was performed by using the agar overlay modification of the disk diffusion assay (23). The following antibiotic disks (Difco) were employed: chloramphenicol (30 μg), nalidixic acid (30 μg), streptomycin (10 μg), sulfathiazole (300 g), and tetracycline (30 μg). Strains identified as *E. coli* that were resistant to one or more antibiotics other than nalidixic acid were regarded as potential donors.

Determination of donor ability. Potential donor strains were assayed for transferability by mating with *E. coli* K-12 C600. The potential donor and recipient were grown separately overnight in nutrient broth (Difco) at 37°C.

After incubation, the potential donor was diluted 1:10 in fresh broth and mixed with the recipient. Matings were allowed to proceed uninterrupted for 24 h in a nonagitating water bath at 30°C. A 0.1-ml portion of the mating mixture was spread on plates of MacConkey agar supplemented with 25 mg of nalidixic acid (Sigma) per ml and 15 μg of the antibiotic to which the potential donor was resistant per ml. Transfer was presumed to have occurred if lactose-negative colonies grew after 24 to 48 h of incubation at 37°C on double inhibitor-supplemented MacConkey agar.

In vitro determination of transfer frequency. Strains MA527 and MA728 were grown overnight in 5 ml of nutrient broth at 37°C. Initial donor density was determined by plating dilutions on MacConkey agar supplemented with 15 μg of tetracycline per ml. A 0.5-ml portion of the overnight donor culture was transferred to 5 ml of fresh nutrient broth and mixed with the 5 ml of recipient. Matings were performed in triplicate in water baths at 5°C intervals from 15 to 35°C. Matings were left uninterrupted for 24 h to increase the likelihood of observing transfer between strains not genetically characterized. Afterward, the mating mixture was serially diluted and spread on double inhibitor-supplemented MacConkey agar supplemented with 15 μg of tetracycline and 25 μg of nalidixic acid per ml. Colonies growing on double inhibitor-supplemented MacConkey agar after 24 h at 37°C were scored as presumptive transconjugants.

Frequency of transfer was calculated as the number of presumptive transconjugants per initial number of

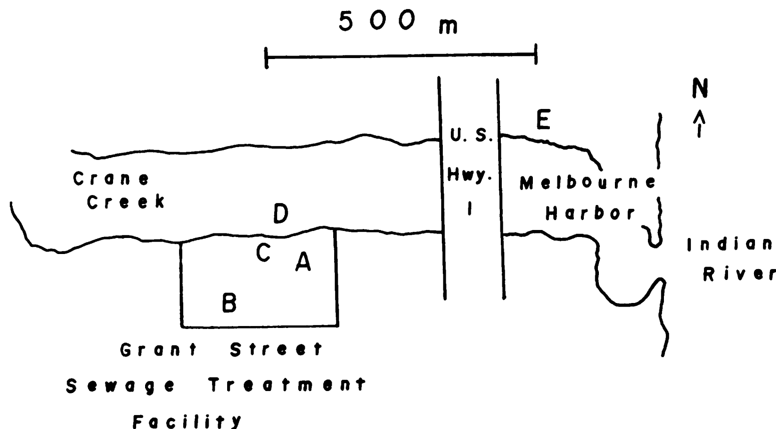

FIG. 1. Study sites: A, residential sewage inflow; B, the degritter tank, mixing point of all local sewage, including that from the regional hospital; C, the chlorinated sewage effluent; D, Crane Creek beside the effluent release point; E, a site approximately 500 m downstream of the effluent release point.

donors (29). Transconjugants were picked and tested for their biochemical characteristics and antibiotic resistance.

In situ determination of transfer frequency. Strains MA527 and MA728 were grown separately overnight in 20 ml of nutrient broth. The cells were centrifuged and washed three times with cold 0.9% phosphate-buffered saline (pH 7.2). After turbidimetric adjustment to 2.5×10^7 coliforms per ml, the cultures were drawn into sterile syringes and transported on ice to the sample site. Before the parental strains were mixed, a sample of each was taken, placed on ice, and returned to the laboratory for determination of initial densities. Membrane diffusion chambers, as described by Fliermanns and Gordon (11) (20-ml volume), were inoculated with 18 ml of the recipient and 2 ml of the donor and then submerged. Matings were allowed to proceed uninterrupted for 24 h and were analyzed as described above.

In situ survival of the parental strains was determined by comparing the initial density with the final density from separate parental control chambers treated in a manner similar to that of the crosses.

Isolation of plasmid DNA. Cleared lysates of the parental and randomly chosen putative transconjugant strains were prepared by the sodium dodecyl sulfate-salt precipitation technique of Guerry et al. (18). Approximately 30 μl of ethanol precipitated DNA from cleared lysates. The DNA was subjected to electrophoresis in a vertical slab gel apparatus containing 0.7% agarose (Bethesda Research Laboratories, Bethesda, Md.), as described by Meyers et al. (25), and compared to standards (22).

RESULTS

The number of coliforms present at the sites examined before the transfer studies (Table 2) varied from practically none at site C, where chlorinated water returns to the stream, to 2.4×10^7 colony-forming units per 100 ml at sites A and B, the raw sewage. At site D, downstream from the effluent release point, the coliform counts ranged from 1.0×10^6 to 8.8×10^6 colony-forming units per 100 ml.

Coliforms resistant to chloramphenicol, streptomycin, and tetracycline (15 μg/ml) were isolated by direct selection at sites A, B, and D (Table 3). Streptomycin-resistant strains occurred most frequently, representing 8.3 to 13.2% of the total coliforms. Tetracycline resistance occurred in 5.0 to 9.3% of the coliforms examined, whereas chloramphenicol resistance was expressed in only 0.6 to 1.7% of the strains tested. The API 20E system identified 78 of the resistant isolates definitively as *E. coli*.

Of the 78 antibiotic-resistant *E. coli* strains biochemically characterized, 31 were chosen for further study based on their ability to transfer their resistance to a restriction- and modification-negative *E. coli* K-12 C600 (Table 4). Of these 31, 9 came from site B (the degritter tank), 11 came from site A (the sewage inflow), and the remainder came from site D (the effluent release point).

Of the seven different antibiotic resistance patterns present among the 31 strains exhibiting antibiotic resistance transfer, four included the combination of tetracycline and streptomycin resistance. On this basis, it was decided that this combination of resistance would be monitored by using MA527 in the in vitro mating studies designed to maximize transconjugant recovery and in the in situ membrane diffusion chamber studies. One of the tetracycline- and streptomycin-sensitive *E. coli* strains isolated in our survey was manipulated to obtain a nalidixic acid-

TABLE 2. Coliform population densities in the Melbourne sewage system

Samples site[a]	No. of samples	Density (colony-forming units per 100 ml)	
		Mean	Range
A	36	2.6×10^7	5.8×10^5–8.3×10^7
B	9	1.7×10^7	3.4×10^6–4.1×10^7
C	5	1.0	0–2.0
D	29	6.1×10^5	1.0–8.8×10^6

[a] Sites: A, pipe entering sewage treatment plant from a residential area; B, sewage treatment facility degritter tank; C, sewage effluent just before release; D, Crane Creek at the effluent release point. See also the legend to Fig. 1.

TABLE 3. Antibiotic-resistant coliform bacteria within the Melbourne sewage system

Sample site[a]	Mean % resistance to:		
	Chloramphenicol	Streptomycin	Tetracycline
A	0.6 ± 0.3	8.3 ± 0.4	5.0 ± 1.5
B	1.7 ± 1.4	10.8 ± 2.6	9.3 ± 2.0
C	1.2 ± 0.5	13.2 ± 3.7	6.7 ± 2.4

[a] See footnote *a* of Table 2 and the legend to Fig. 1.

TABLE 4. Patterns of resistance and occurrence of transfer in environmental isolates of *E. coli*

Resistance pattern[a]	No. of strains	No. exhibiting transfer[b]
Sm, Tc	19	12
Sm, St, Tc	24	12
Cm, Sm, St, Tc	13	3
Cm, Sm, St	5	0
Sm	4	0
Sm, St	3	1
St, Tc	3	0
Tc	3	1
Cm, Sm, Tc	2	1
Cm, St	2	1

[a] Cm, chloramphenicol; Sm, streptomycin; St, sulfathiazole; Tc, tetracycline.

[b] Represents the number of strains of a particular resistance pattern capable of transferring their resistance to C600.

resistant clone, MA728, which grew on nutrient agar supplemented with 50 μg of nalidixic acid per ml. This strain was subsequently used as the recipient for in vitro and in situ mating studies.

In vitro matings between MA527 and MA728 demonstrated that the acquisition of tetracycline resistance by MA728 varied with temperature (Fig. 2). The frequency of antibiotic resistance transfer was greatest at 25°C (1.8×10^{-4}) and lowest at 35°C (1.5×10^{-7}). The transfer frequencies for matings performed at 15, 20, and 30°C were 4.3×10^{-6}, 1.6×10^{-4}, and 4.4×10^{-5}, respectively.

In situ, the mating frequencies for MA527 and MA728 at site B also varied with temperature (Table 5). The frequency of tetracycline resistance transfer was 3.2×10^{-5} at 22.5°C and 1.0×10^{-6} at 29.5°C.

When tetracycline-resistant clones were scored for additional markers, all were found to carry streptomycin resistance as well. No transconjugants were present in matings performed at site E, regardless of the temperature.

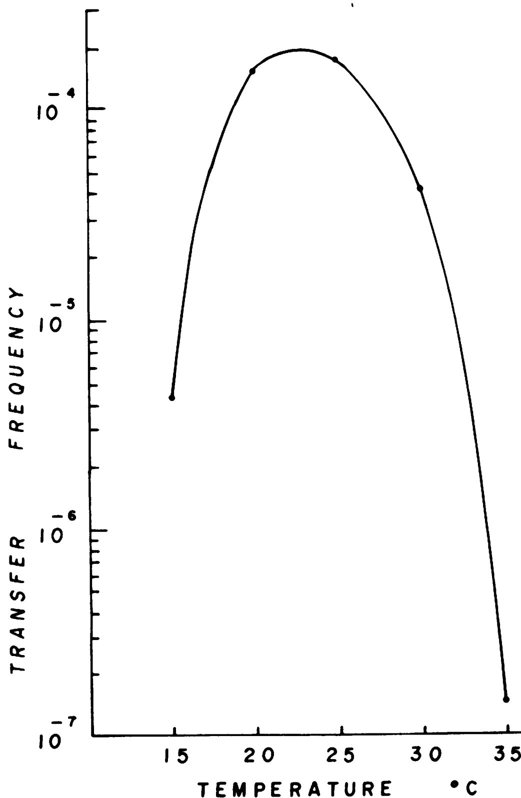

FIG. 2. Antibiotic resistance transfer frequencies at various temperatures in in vitro matings between MA527 and MA728. Data points represent mean values from multiple matings.

TABLE 5. In situ transfer frequencies

Sample site[a]	Date	Temp (°C)	Transfer frequency[b]
B	February 1981	22	3.2×10^{-5}
B	July 1981	29.5	1.0×10^{-6}
E	February 1981	18	$<3.1 \times 10^{-8}$
E	July 1981	28.5	$<7.6 \times 10^{-8}$

[a] See footnote *a* of Table 2 and the legend to Fig. 1.
[b] Calculated as the number of presumptive transconjugants per initial number of donors per milliliter.

Fourteen transconjugants subjected to biochemical characterization were identical to the recipient MA728. All 14 revealed antibiotic resistance patterns identical to those of MA527 in addition to the nalidixic acid resistance of the recipient. All multiply resistant transconjugants from in vitro and in situ matings, as well as MA527, were shown to bear a 60-megadalton plasmid, pKK1 (Fig. 3). No large plasmid was observed in the recipient, MA728.

DISCUSSION

The enumeration of coliform bacteria in and around the municiple sewage treatment system (Table 2) was done to establish the correct cell densities to use in the membrane diffusion chamber studies. The number of antibiotic-resistant coliforms found (Table 3) is not surprising when one recognizes that this system handles sewage

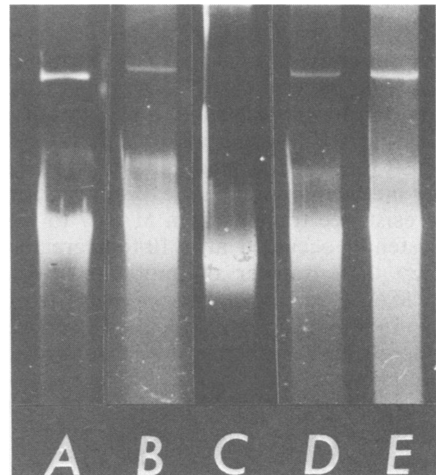

FIG. 3. Electrophoretic analysis of minilysates. A, *E. coli* JS with size reference covalently closed circular R plasmid R1 (60 megadaltons); B, *E. coli* donor strain MA527 with R plasmid pKK1 (60 megadaltons); C, *E. coli* recipient strain MA728 with no plasmid; D and E, transconjugants from in situ cross of MA527 and MA728.

from a hospital and a college campus, as well as a number of residential areas. The stream into which the effluent from the plant returns drains grazing land and therefore is another source of antibiotic-resistant organisms. Isolates resistant to streptomycin, tetracycline, and chloramphenicol (15 μg/ml) were common at all locations except site C, the sewage effluent release point. Among these strains, 78 were identified as *E. coli* and were thought to represent a pool of potential R plasmid donors typical of the environment.

The 78 antibiotic-resistant strains were screened for resistance transfer in vitro at 30°C, a temperature observed at the various sampling sites at different times of the year and within the range cited as optimal for plasmid transfer (35). The fact that only 40% of the resistant strains expressed their transfer potential in our screening by no means precludes the possibility that a greater number might transfer under different conditions or with another recipient. It is also probable that some of our isolates bear certain resistance traits that have a chromosomal origin and are probably genetically regulated to repress transfer.

Since multiply resistant strains with transferability were randomly encountered with almost equal frequency in sewage entering the plant, sewage within the plant, and water downstream of the plant, we must conclude that there are multiple sources of resistance traits. The observation of seven distinct antibiotic resistance patterns among the resistance transfer-positive strains is indicative of multiple sources of R plasmids as well.

An *E. coli* strain (MA527) isolated from study site A was chosen as the donor for in vitro and in situ mating experiments. It expresses transferability of its tetracycline and streptomycin resistance phenotypes to *E. coli* K-12 C600 as well as to strain MA728, originally isolated from study site E. In preliminary experiments, antibiotic resistance transfer from MA527 to MA728 consistently occurred at a 10-fold greater frequency than transfer of antibiotic resistance from MA527 to C600 at a variety of temperatures in vitro; therefore, MA728 was chosen as the recipient for further in vitro matings and the in situ matings.

The thermosensitivity of transfer in both the in vitro and in situ matings in the sewage is in agreement with the results of Smith et al. (32). This phenomenon increases the potential for R plasmid dissemination in aquatic systems.

The maintenance or expression of an R plasmid under nutrient-deficient conditions may not have any selective advantage to the host under such stress and may in fact be a burden. Nutrient concentrations in the effluent-receiving waters are less than in raw sewage, and the salinity is 10 times higher, providing a more stressful environment. The failure of antibiotic resistance to be transferred in the sewage effluent-receiving waters may be attributable to the lack of nutrients, increased salinity, and the resultant physiological injury of the bacteria. Subjecting physiologically injured recipients to inhibitor-containing media may be too stressful for their recovery, as suggested by Bissonnette et al. (3).

Periodically, high numbers of coliforms are found in the effluent-receiving waters (Table 2). Although no R plasmid transfer was detected there, the occurrence of transferable resistance is observed in coliforms isolated from these waters. This suggests that conditions may transiently exist for R plasmid transfer in the downstream area.

To our knowledge, this work is the only in situ study of antibiotic resistance transfer with membrane diffusion chambers. The recombination frequencies recorded dispute the conclusion of Linton et al. (21) that the conditions for R plasmid-mediated conjugation are not common to sewage.

Future studies with membrane diffusion chambers should provide information on the energy requirements and other factors involved in R plasmid maintenance and transfer in situ. In addition, similar studies in sewage-contaminated sediments and less saline sewage effluent-receiving waters will determine the roles of these environments in the dissemination of R plasmids among fecal bacteria.

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