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Full Length Research Paper

Intergenic and intragenic conjugal transfer of multiple antibiotic resistance determinants among bacteria in the aquatic environment of Bangladesh

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Conjugation process was conducted to determine the means of transferring multiple loci of antibiotic resistant gene among bacteria in the surface water of Bangladesh. Our observation demonstrated that tetracycline resistant gene can be spread between bacteria in the aquatic environment. In this study, it was surprisingly observed that tetracycline resistant gene was transferable horizontally by conjugation process from *Vibrio cholerae* to *Esherichia coli* (intergenic) and *E. coli* to *E. coli* (intragenic) in combination with sulphamethoxazole-trimethoprim (SXT), streptomycin and erythromycin as a self transposable tetracycline element. In intergenic transfer, conjugation frequency was more than intragenic transfer. Frequencies of transfer generally ranged from 10^{-5} to 10^{-3} transconjugants per input donor and recipient cell. The results of these experiments suggested that the mobility of the antibiotic resistance determinants may further contribute to the development and spread of antibiotic resistance among pathogenic bacteria, particularly since antibiotics are indiscriminately used in Bangladesh.

Key words: Conjugation, gene transfer, aquatic environment.

INTRODUCTION

From the beginning of the production of antibiotics in 1930, antibiotic resistant bacteria have been on the rise. Bacteria become resistant to the antibiotics so quickly that the antibiotic productions of new derivatives are very difficult. Recently, we have seen many old-time diseases reemerge with multi-drug resistances, making treatment very difficult and costly. Due to the rise of multi-drug resistant bacteria, scientists are trying to realize how so many different strains of bacteria could have acquired this gene so quickly. In many virulent strains of bacteria, pathogenicity islands are found, but their mode of action is largely unknown (Hochhut and Waldor, 1999). Acquisition of genetic material among microorganisms has been known for almost 50 years (Avery et al., 1944). Phages and transposons are also able to transfer antibiotic resistances to many bacterial strains. Transduction (bacteriophage-mediated transfer [Kokjohn, 1989]) and conjugation (transfer by direct contact between donor and recipient cells were later observed as transformation (uptake of extracellular DNA [Stewart, 1989]) of genetic material. Within last 25 years, new elements called conjugative tansposons, plasmids, and related elements have been discovered. In 1999, Hochhut and Waldor proposed the term constin for all elements that have a conjugal mode of transfer, are self-transmissible to the recipient and integrate into the host chromosome (Hochhut and Waldor, 1999).

Antibiotic resistance may be acquired by mutation and selection, with passage of the trait vertically to daughter cells. More commonly, resistance is acquired by horizontal transfer of resistance determinants from a donor cell, often of another bacterial species, by transduction, transformation, or conjugation. Horizontal gene transfer,

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the intraspecies and interspecies exchange of genetic information, plays an important role in the evolution of bacteria (Cruz and Davies 2000; Jain et al., 1999; Krishnapillai, 1996; Ochman et al., 2000; Syvanen, 1994). Resistance that is acquired by horizontal transfer can become rapidly and widely disseminated either by clonal spread of the resistant strain itself or by further genetic transfers from the resistant strain to other susceptible strains. The horizontal transfer of integrons is considered as the most efficient means for dissemination of resistance genes and emergence of multiresistant strains (Chandler and Claverys, 2001; Hanau-Bercot et al., 2002; Rowe-Magnus and Mazel, 2001). Besides these bits of information, we were interested in finding out the way of transmitting genetic material in the environmental water collected from different surveillance sites including rivers, ponds and lakes of Bangladesh. This study also suppor-ted the horizontal gene transfer process that the multiple antibiotic resistant determinants including tetracycline, streptomycin, sulfamethoxazole-trimethoprim and erythromycin were competent for intergenic transfer between *Vibrio cholerae* and *Esherichia coli* and intragenic transfer between *E. coli* species.

MATERIALS AND METHODS

Bacterial strains and media

The bacterial strains used in this study are described in a Table 1. *V. cholerae* were routinely maintained on Luria-Bertani and gelatin agar plate and *E. coli* were maintained on MacConkey agar plate. Agar (Difco) was added to give a final concentration of 1.5% when a solid medium was desired. Cultures were grown at 37°C unless indicated otherwise. Occasionally antibiotics were added into this medium. Bacterial strains were tested for their susceptibility to tetracycline 15 µg/ml, nalidixic acid 15 µg/ml, kanamycin 50 µg/ml, gentamicin 15 µg/ml, ampicillin 75 µg/ml, streptomycin 25 µg/ml, chloramphenicol 10 µg/ml, sulfamethoxazole 23.75 µg/ml, trimethoprim 32 µg/ml. All of these antibiotics were obtained from Sigma Chmical Co.

Chromosomal DNA extraction

Chromosomal DNA isolation was carried out by the method described by Maniatis et al. (1989). 50 mM glucose, 25 mM lysozyme and proteinase K were used to lyse bacterial cell wall from overnight cultures. The preparation was extracted with phenol and then with phenol-chloroform-isoamylalcohol (25:24:1) followed by a precipitation with double volume of absolute ethanol. The precipitated DNA then spin for 5 min in the centrifuger. The DNA pellet was then dried in a vacuum drier and dissolved in 400 µl of TE, and treated with RNase A. Finally DNA was prepared after extraction with both phenol and phenol-chloroform-isoamylalcohol followed by double volume ethanol precipitation as before.

Probes and hybridization

For preparation of DNA blots, total cellular DNA was isolated from overnight cultures. Five-microgram aliquots of DNA were digested with the appropriate restriction enzymes (Bethesda Research Laboratories [BRL], Gaithersburg, Md.), electrophoresed in 0.8% agarose gels, and blotted onto nylon membranes (Hybond; Amersham) by Southern blotting (Southern, 1975). The SXT probe used in this study to detect tetracycline resistant genetic element was a NotI fragment of pSXT1 (Waldor et al., 1996). The probes were labeled by random priming (Feinberg and Volgelstein, 1984) with a random primer DNA labeling kit (BRL) and [-32P]dCTP (3,000 Ci/mmol) (Amersham). Southern blots were hybridized with the labeled probes, and autoradiographs were developed (Faruque et al., 1997; Feinberg and Volgelstein, 1984).

Conjugation experiments

Multidrug-resistant *V. cholerae* and *E. coli* were used for conjugation. In this technique, both the donor and recipient strains were grown in LB. Cultures were dispensed as follows: 0.4 ml donor + 4.0 ml recipient for transformation, 0.4 ml donor + 3.6 ml LB, 4.0 ml of recipient as control. Mixed cultures were centrifuged, supernatant aspirated and pellets were resuspended in 0.3 ml LB. Using sterile Pasteur pipette, cell suspensions were transferred to nitrocellulose paper on TSA plate for mating. Plates were incubated overnight at 37°C. After incubation, nitrocellulose papers were transferred to containers containing 10 ml of LB. Containers were vortexed and 100 µL suspension spread on selection plate that was allowed to grow only transconjugant. Plates were incubated overnight at 37°C and checked for the growth of transconjugant. Presumptive transconjugants were confirmed by the biochemical and API test. Transfer of antimicrobial resistant genes was confirmed by standard antibiotic disks and southern hybridization.

Antimicrobial resistance

All transconjugants were tested for antimicrobial resistance by the method of Bauer et al. (1966) with standard antibiotic disks (Oxoid Ltd., Basingstoke, Hampshire, United Kingdom) at the following antibiotic concentrations (in micrograms per disk): ampicillin, 10; chloramphenicol, 30; streptomycin, 10; tetracycline, 30; trimethoprim-sulfamethoxazole (SXT), 1.25 and 23.75, respectively; kanamycin, 30; gentamicin, 10; nalidixic acid, 30 (Faruque et al., 1999).

RESULTS

Tetracycline resistant gene transfer by conjugation method

Tetracycline resistant gene can be transferred between intraspecies and interspecies. In this study, surprisingly it was observed that tetracycline resistant gene was transferred horizontally in combination with SXT, streptomycin and erythromycin and that is the reason it can be called self transposeable tetracycline element. This gene can be transmitted from *V. cholerae* to *E. coli* (Table 2) and *E. coli* to *E. coli* (Table 3). After analyzing 15 *V. cholerae*, two starins were capable of transferring the resistant gene and out of 21 *E. coli* strains only one had the same capacity (Table 1). According to the result, the transfer frequency from *V. cholerae* to *E. coli* is fifty times than from *E. coli* to *E. coli*. The transfer and transformed frequency for both cases are shown in Tables 2 and 3.

Table 1. Bacterial strains used for this study.

Bacterial strain	Chromosomal phenotypes	Source	Capability to transfer resistant gene
V. cholerae 1455	Tet^R, Nal^R, E^R, SXT^R,S^R	Environmental Water	-
V. cholerae 1456	Tet^R, Nal^R, E^R, SXT^R,S^R	Environmental Water	-
V. cholerae 1464	Tet^R, Nal^R, E^R, SXT^R,S^R	Environmental Water	-
V. cholerae 1465	Tet^R, Nal^R, E^R, SXT^R,S^R	Environmental Water	-
V. cholerae 1466	Tet^R, Nal^R, E^R, SXT^R,S^R	Environmental Water	-
V. cholerae 1467	Tet^R, Nal^R, E^R, SXT^R,S^R	Environmental Water	-
V. cholerae 1470	Tet^R, Nal^R, E^R, SXT^R,S^R	Environmental Water	-
V. cholerae 1473	Tet^R, Nal^R, E^R, SXT^R,S^R	Environmental Water	+
V. cholerae 1475	Tet^R, Nal^R, E^R, SXT^R,S^R	Environmental Water	+
V. cholerae 1476	Tet^R, Nal^R, E^R, SXT^R,S^R	Environmental Water	-
V. cholerae 1478	Tet^R, Nal^R, E^R, SXT^R,S^R	Environmental Water	-
V. cholerae 1481	Tet^R, Nal^R, E^R, SXT^R,S^R	Environmental Water	-
V. cholerae 1483	Tet^R, Nal^R, E^R, SXT^R,S^R	Environmental Water	-
V. cholerae 1487	Tet^R, Nal^R, E^R, SXT^R,S^R	Environmental Water	-
V. cholerae 1514	Tet^R, Nal^R, E^R, SXT^R,S^R	Environmental Water	-
E. coli 103	Tet^R, Nal^R, E^R, SXT^R,S^R	Environmental Water	-
E. coli 104	Tet^R, Nal^R, E^R, SXT^R,S^R	Environmental Water	-
E. coli 106	Tet^R, Nal^R, E^R, SXT^R,S^R	Environmental Water	-
E. coli 108	Tet^R, Nal^R, E^R, SXT^R,S^R	Environmental Water	-
E. coli 109	Tet^R, Nal^R, E^R, SXT^R,S^R	Environmental Water	-
E. coli 112	Tet^R, Nal^R, E^R, SXT^R,S^R	Environmental Water	-
E. coli 113	Tet^R, Nal^R, E^R, SXT^R,S^R	Environmental Water	-
E. coli 115	Tet^R, Nal^R, E^R, SXT^R,S^R	Environmental Water	-
E. coli 118	Tet^R, Nal^R, E^R, SXT^R,S^R	Environmental Water	-
E. coli 120	Tet^R, Nal^R, E^R, SXT^R,S^R	Environmental Water	-
E. coli 122	Tet^R, Nal^R, E^R, SXT^R,S^R	Environmental Water	-
E. coli 125	Tet^R, Nal^R, E^R, SXT^R,S^R	Environmental Water	-
E. coli 126	Tet^R, Nal^R, E^R, SXT^R,S^R	Environmental Water	-
E. coli 127	Tet^R, Nal^R, E^R, SXT^R,S^R	Environmental Water	-
***E. coli* 130**	Tet^R, Nal^R, E^R, SXT^R,S^R	Environmental Water	+
E. coli 133	Tet^R, Nal^R, E^R, SXT^R,S^R	Environmental Water	-
E. coli 135	Tet^R, Nal^R, E^R, SXT^R,S^R	Environmental Water	-
E. coli 136	Tet^R, Nal^R, E^R, SXT^R,S^R	Environmental Water	-
E. coli 138	Tet^R, Nal^R, E^R, SXT^R,S^R	Environmental Water	-
E. coli 139	Tet^R, Nal^R, E^R, SXT^R,S^R	Environmental Water	-
E. coli 140	Tet^R, Nal^R, E^R, SXT^R,S^R	Environmental Water	-
E. coli DH5α	Amp^R, Kan^R	Laboratory strain	Recipient

Tet, Nal, Kan, Amp, S, E, Sxt represents tetracycline, nalidixic acid, kanamycin, ampicillin, streptomycin, erythromycin and sulphamethoxazole-trimethoprim, respsctevely and R indicates resistant, (+) for gene transformation and (-) for inability to transfer gene.

Waldor et al. (1996) published in a paper that conjugative transposon-like SXT element could be conjugally transferred from *V. cholerae* O139 to *V. cholerae* O1 and *E. coli* strains, where it integrated into the recipient chromosomes in a site-specific manner independent of recA (Waldor et al., 1996).

Detection of transmissible tetracycline transposon element

Tetracycline element contains tetracycline, sulfamethoxazole-trimethoprim, streptomycin and erythromycin. In this study, for the detection of transfer-

Table 2. Intergenic transfer of tetracycline resistant gene.

Donor		Recipient		Transconjugant chromosomal phenotypes	Conjugation Frequency	
Strain	Chromosomal phenotypes	Strain	Chromosomal phenotypes		Transfer frequency	Transformed frequency
VC01 ogawa, 1473	Tet^R, S^R,Sxt^R, Nal^R, E^R, Fr^R	DH5α Pmsf8.0	Amp^R,Kan^R	Tet^R,S^R,Sxt^R,E^R,Amp^R, Kan^R, Nal^R	$2.3x\ 10^{-4}$	$9.09x\ 10^{-5}$
VC01 ogawa, 1475	Tet^R, S^R,Sxt^R, Nal^R, E^R, Fr^R	DH5α Pmsf8.0	Amp^R, Kan^R		2.7x 10-4	

Tet, Nal, Kan, Amp, S, E, Fr, Sxt represents tetracycline, nalidixic acid, kanamycin, ampicillin, streptomycin, erythromycin, furazolidone and sulphamethoxazole-trimethoprim, respectively, and R indicates resistant.

Table 3. Intragenic transfer of tetracycline resistant gene.

Donor		Recipient		Transconjugant chromosomal phenotypes	Conjugation Frequency	
Strain	Chromosomal phenotypes	Strain	Chromosomal phenotypes		Transfer frequency	Transformed frequency
E. coli 130	Tet^R, S^R,Sxt^R, Nal^R, E^R	DH5α Pmsf8.0	Amp^R, Kan^R	Tet^R,S^R,Sxt^R, Nal^R,E^R,Amp^R, Kan^R	$4.7x\ 10^{-3}$	$4.3x\ 10^{-3}$

The frequency of conjugation was calculated by dividing the number of transconjugant cells by the total number of donor and recipient cells. In transfer frequency, donor bacteria and in transformed frequency, recipient bacteria were used to calculate conjugation frequency. Tet, Nal, Kan, Amp, S, E, SXT represents Tetracycline, Nalidixic acid, Kanamycin, Ampicillin, Streptomycin, Erythromycin and Sulphamethoxazole-Trimethoprim, respectively, and R indicates resistant.

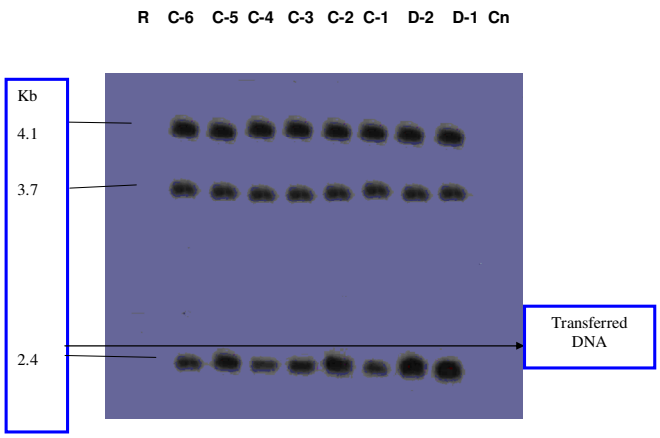

Figure 1. Southern hybridization analysis of *Bgl*I digested chromosomal DNA of donor, conjugant and recipient of tetracycline resistant gene. SXT probe was used for hybridization. Lanes: Cn, negative control; D, donor (D-1: *V. cholerae* and D-2: *E. coli*); C, conjugant (C-1, C-2 and C-3 are conjugants of intergenic and C-4, C-5, C-6 are conjugants of intragenic transfer); R, recipient (*E.coli* DH5α). The size of the restriction fragments of transferred DNA are indicated in the figure.

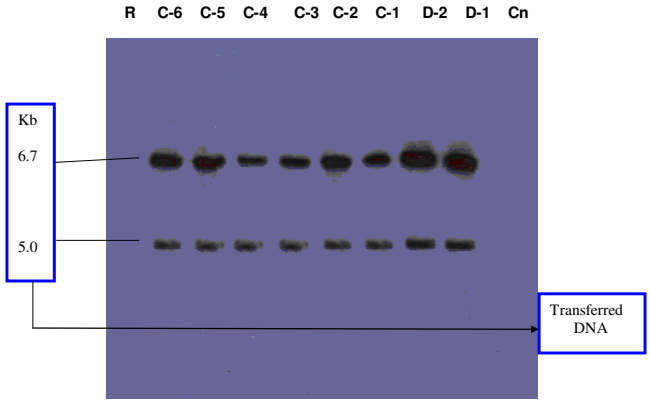

Figure 2. Southern hybridization analysis of *Hind*III digested chromosomal DNA of donor, conjugant and recipient of tetracycline resistant gene. SXT probe was used for hybridization. Lanes: Cn, negative control; D-, donor (D-1: *V. cholerae* and D-2: *E. coli*); C, conjugant (C-1, C-2 and C-3 are conjugants of intergenic and C-4, C-5, C-6 are conjugants of intragenic transfer); R, recipient (*E. coli*DH5α). The restriction fragments of transferred DNA are indicated in the figure.

red tetracycline resistant element, southern hybridization were performed by the restriction digestion of the chromosomal DNA of donor, transconjugants and recipient by *Bgl*I (Figure 1) and *Hind*III (Figure 2) and hybridized with sulphamethoxazole-trimethoprim (SXT) probe. It revealed that digested band patterns of tetracycline resistant element of different molecular weight were transferable. The transferred fragment of tetracycline resistant gene are indicated in the blot. To identify the

genetic changes in transconjugant associated with the tetracycline resistant gene, cloned SXT gene probe were used to confirm whether self transposeable tetracycline element contains SXT gene or not. Three different *Bgl*I (Figure 1) restriction patterns and two *Hind*III (Figure 2) restriction patterns of the transmitted tetracycline element were observed in the transconjugant that were similar with the restriction fragments of donor chromosomal DNA.

DISCUSSION

Our study suggested that tetracycline resistant gene can be spread between bacteria in the aquatic environment. In this study, it was observed that tetracycline resistant gene was transferable horizontally by conjugation process in combination with sulphamethoxazole-trimethoprim (SXT), streptomycin and erythromycin as a self transposeable tetracycline element. By hybridization with SXT probe, this result was confirmed. DNA-DNA hybridization experiments revealed that the SXT probe shared sequence homology with all of the *Bgl*I and *Hind*III fragments present in donor and transconjugant. Tetracycline resistant gene can be transferred from *V. cholerae* to *E.coli* and *E.coli* to *E.coli* according to our analysis. In a specific recognition site in the host genome, transposeable element became integrated. It was published that SXT element that contains sulphamethoxazole-trimethoprim and streptomycin was able to transfer chromosomal DNA from the donor to recipient by horizontal transformation (Hochhut et al., 2000; Waldor et al., 1996). We found that intragenic transfer of resistant determinants occurred between *E. coli* to *E. coli* DH5α, but it was at a frequency of less than intergenic transfer which occurred between *V. cholerae* as a donor and *E. coli* DH5α as a recipient. Analysis of transconjugants by agarose gel electrophoresis verified the acquisitions of tetracycline element by *E. coli* DH5α. DNA preparations of transconjugants were obtained and digested with restriction enzymes. The resulting DNA fragments that were obtained matched the migration distances of donor DNA. Three *Bgl*I and two *Hind*III DNA fragments of transferred DNA which closely matched donor DNA fragments are shown in the figures.

Transformation of gene can be reflected by two ways on the nature by showing either positive or negative impact. Tamar Barkay and his coworkers reported that gene transfer contributes to acclimation for the detoxification and degradation of pollutants (Barkay and Pritchard, 1988; Spain et al., 1980). Although the central role of enrichment in acclimation cannot be disputed (Chen and Alexander, 1989), horizontal gene transfer may be a principal mechanism to promote metabolic diversity in acclimated communities. Another application of the ability to detect gene transfer in indigenous microbial communities is in assessing the risk of genetically engineered microorganisms (GEMs) in the environment. The prospect of using GEMs in environmental management raises the issue of gene transfer (Halvorson et al., 1985; Klingmfiller 1988). In contrast, our findings focused on the dissemination of multiple antibiotic resistant bacteria in the aquatic environment and the mobility of their antibiotic resistance determinants may further contribute to the development and spread of antibiotic resistance among pathogenic bacteria, particularly since antibiotics are indiscriminately used in Bangladesh and thus the antibiotics is being threatened by the emergence of increasingly resistant bacteria.

The horizontal transfer of genes in prokaryotes is a major evolutionary driving force, and it has been estimated that 20% of *E. coli* genes came from other organisms. In an article published in Nature, John Beaber and colleagues at Tufts University School of Medicine report that the SOS response-induction of specific genes in response to DNA damage—alleviates the repression of genes necessary for horizontal transfer of the mobile integrating conjugative element, SXT. This is a ~100 kb plasmid derived from *V. cholerae* that confers resistance to the antibiotics chloramphenicol, trimethoprim, streptomycin and methoxazole. Most of the *V. cholerae* isolates were resistant to SXT element; acquisition is by spreading (Beaber et al., 2004).

Conjugation is generally mediated by plasmids. When conjugation is initiated via a mating signal, a complex of proteins called the relaxosome creates a nick in one plasmid DNA strand at the origin of transfer, or *oriT*. The lack of conjugal transfers might be explained by the inability to form mating pairs and to obtain the proper cell-to-cell contact or to host restriction modification systems (Gonzalez and Kunka, 1983). If the F-plasmid becomes integrated into the host genome, donor chromosomal DNA may be transferred along with plasmid DNA. The amount of chromosomal DNA that is transferred depends on how long the bacteria remain in contact. The transferred DNA can be integrated into the recipient genome via recombination. When conjugative transposons are transferred to another host, they excise from the chromosome and form an extra chromosomal circular form. To determine if the SXT element also had a circular intermediate, a PCR primer that would only recognize the joined ends of the element was made. Indeed, a circular intermediate was found. When SXT excises form the chromosome, it does not leave a copy of the element behind. Excision was not be recA dependant, because a recA strain still contained the circular intermediate (Hochhut and Waldor, 1999). Therefore, recA must be required for transfer of the element to the recipient.

The horizontal transfer of antibiotic resistance genes from the gastrointestinal tracts of domestic animals to the gastrointestinal tract of humans remains a controversial subject (Macovei and Zurek, 2006) because the ecology

and horizontal transfer of antibiotic resistance and virulence determinants in the environment are poorly understood. But in our study, conjugal transfer to aquatic bacteria was successfully detected by the formation of a new phenotype. Experiments were performed under optimal conditions using laboratory incubations. Under these conditions, transfer was readily measurable, indicating that recipients are present in natural waters and formation of transconjugants with new phenotypes resulting in the development and spread of antibiotic resistance among pathogenic bacteria, particularly since antibiotics are indiscriminately used in Bangladesh without proper prescription.

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