ONE ORGANISM'S WASTE IS ANOTHER ORGANISM'S GENE POOL: GENE TRANSFER IN MARINE ENVIRONMENTS

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"If you gotta lot of knives and forks,
You gotta find something to cut."
-Bob Dylan

Abstract:

Gene transfer is ubiquitous in nature, and may play important roles in gene flow and evolution. The goal of this independent project has been to evaluate the efficiency of conjugational transfer of DNA between bacterial species under simulated marine conditions.

Attempts to use a broad host range transposon delivery assay (a narrow host range plasmid carrying Tn4351 and tetracycline resistance) for gene transfer were unacceptable due to low rates of transposition in *E. coli* recipients, and the lack of detectable transposition in a genetically marked marine *Vibrio* species. As a result, plasmid transfer between genetically marked *E. coli* strains under specific marine-like environments was used as an assay for conjugational transfer of genetic information.

Relatively high levels of plasmid transfer (ca. 10^{-2} - 10^{-3} per recipient cell) were observed under the following conditions: standard agar-based spot matings (both LB and marine salts media), suspended in filter sterilized seawater, on a common marine surface (bivalve shell), and within the intestinal tract of the quahog, a common edible filter feeding marine bivalve (*Mercenaria mercenaria*).

These results strongly suggest that enteric bacteria entering a variety of marine environments, including the gut tract of edible shellfish, are capable of transferring genetic information to other bacteria. This may have implications for the continued spread of antibiotic resistance among bacteria, as well as the ultimate fate of genetically engineered microbes within the marine environment.

INTRODUCTION:

Gene transfer between bacteria, once thought to be essentially a laboratory artifact, has been shown to occur throughout nature in a variety of environments (3, 13, 20). Genetic material can be transferred between different genera of bacteria by three main strategies, all of which have been observed to occur under natural conditions: transduction, transformation, and conjugation (20). This concept becomes increasingly important in light of regulatory concerns with the release of genetically engineered microorganisms into the environment, and the ability of such organisms to contribute their new traits to other, wild microorganisms.

With the rise of multiply antibiotic resistant pathogenic bacteria due to horizontal transfer of resistance genes, these concerns take on medical importance. It has been shown that antibiotic resistance genes are indeed capable of transfer between bacteria in many environments that bring such organisms into close proximity (13). These mobile antibiotic resistance genes can exist on chromosomes, plasmids, and transposable elements (13, 19). Natural transformation has been shown to allow antibiotic resistance genes to move into "wild" bacteria under natural conditions (15, 16, 17). Conjugational transfer of such genes has been shown to occur between bacterial species in the animal colon (14), during bacterial infections (11), within nematodes (1), cutworms (2), and even earthworms (8). With the frequent flow of untreated sewage into marine environments, the possibility of bacteria containing mobile resistance elements transferring such genes into marine microorganisms becomes a very real concern.

In this preliminary investigation, gene transfer will be evaluated under a number of conditions designed to mimic natural marine environments. As a self-mobilizing broad host range plasmid was not available in this course, gene transfer will be measured using transposition of the tetracycline and erythromycin resistant, extremely broad host range element Tn4351 (N. Shoemaker, personal communication), as well as the ability of the plasmid carrying the transposon to replicate in permissive host bacteria.

The assay for gene transfer will be carried out with two bacterial donor-recipient systems: between two *E. coli* strains, and between an *E. coli* donor and a marked strain of a recently isolated marine *Vibrio* species. Conditions will include spot mating on marine medium, mating within the

water column, mating on marine surfaces, and conjugation within the gut of a filter feeding marine invertebrate.

The goals of this preliminary study are thus twofold. Can Tn4351 be transferred at detectable frequency from one *E. coli* strain to another *E. coli* strain under marine conditions, including within the gut of a feeding bivalve (or can plasmid transfer be demonstrated in such circumstances)? Additionally, can Tn4351 be transferred from an *E. coli* donor to a specific (recently isolated) marine *Vibrio* species under those conditions as well?

MATERIALS AND METHODS:

Bacterial strains, plasmids, and animals used. L1 is a brightly luminescent marine vibrio obtained from Elena Barbieri as part of an enrichment from a *Limulus* exoskeleton. L1 grew well on standard marine microbiological medium, including LBS and ASW. L1 was rendered resistant to rifampicin (100 ug / ml) and nalidixic acid (40 ug / ml) by spontaneous stepwise mutation.

The *E. coli* transposon donor strain, BW19851 / pEP4351, was constructed and has characteristics as follows. The bacterial strain was constructed by William Metcalf in Barry Wanner's laboratory (W. Metcalf, personal communication), and is based on S17-1. It contains the plasmid transfer genes of RP4 inserted into the bacterial chromosome and the *pir* gene of pR6K in the *uid*A gene. pEP4351, constructed in the Salyers laboratory (personal communication), is a 10.5 kb plasmid containing the pR6K origin of replication, the *mob* site of RP4, chloramphenicol resistance, and Tn4351 (which encodes erthryomycin and tetracycline resistances). The chromosomal *tra* genes in BW19851 provides the gene products in *trans* necessary to promote transfer of pEP4351 (and the plasmid carrying the transposon) into diverse recipient microorganisms.

The *E. coli* recipient strains used were EM24NR and SY327 (N. Shoemaker, personal communication). EM24NR, which lacks the *pir* gene necessary to allow the replication of pEP4341 once introduced, is resistant to both nalidixic acid and rifampicin. SY327 contains the *pir* gene on a lambda lysogen (allowing the replication of pEP4341), and is also resistant to nalidixic acid and rifampicin.

The common marine bivalve called the quahog, *Mercenaria mercenaria*, (as well as advice on subsequent experimental manipulations) was obtained from Edward Enos and other staff of the Marine Resources

Center of the Marine Biological Laboratories, Woods Hole, Massachusetts.

Media and growth conditions. All bacteria were cultivated on LBS, composed of standard Luria Bertani medium with a total of 20 g / liter of NaCl. This medium allows growth of *E. coli* and many marine bacteria, including vibrio strain L1. Solid LBS medium contained 15 g / liter agar. *E. coli* strains were grown at 37°C, while marine vibrio strains were grown at 30°C. LBS medium was supplemented as necessary with various sterile antibiotics (rifampicin at 100 ug / ml, nalidixic acid at 40 ug / ml, and tetracycline at 10 ug / ml) to determine donor, recipient, and transconjugant numbers and frequencies. Artificial seawater medium (ASW) medium was prepared as described by P. Dunlap (instructional material, Microbial Diversity Course, 1996).

Conjugational methods:

1. **Standard protocol.** This procedure follows most standard Gram negative conjugational protocols, such as described by Beringer (6). In a standard conjugation, donor and recipient cells were washed of antibiotics in sterile LBS by centrifugation, and 10 ul of donor and recipients (along with controls) carefully spotted onto the surface of a very dry LBS (or appropriate medium) plate. After overnight incubation at 30°C., the mating spot was resuspended in LBS, washed twice by centrifugation, and dilutions plated on selective medium.

Transfer of plasmids or transposition was monitored by plating dilutions on LBS containing nalidixic acid, rifampicin, and tetracycline, as well as LBS supplemented with nalidixic acid and rifampicin; the latter determines the number of possible recipient cells in the mating spot, while the former the number of successful transconjugants.

Mock spot matings with donor or recipient only were also carried out, resuspended, and plated on selective medium in order to determine non-exconjugant background colonies present. Controls were clean of false positive colonies at the dilutions and drug concentrations used in these experiments.

2. **Water column.** This procedure was modified from that of Paul, et al, 1991 (15, 16). Donor and recipient cells were prepared as described above, then 100 ul of each added to 10 ml of filter sterilized

seawater. After overnight incubation at 30°C. with periodic agitation on a rocker platform, a 1 ml aliquot was removed, centrifuged to collect the cells, washed twice, and dilutions plated on selective medium as described above.

3. **Submerged natural substrate.** This procedure was modified from that of Bale, et al, 1988 and others (4, 17, 18). Donor and recipient cells were prepared as described above. Bivalve shells that had been scrubbed, washed in distilled water, and autoclaved were used as a substratum for conjugation. Donor and recipient cells (100 ul of each) were mixed, added to the interior of a shell, and placed onto a piece of filter paper moistened with seawater in a large petri dish. The "moist chamber" was left overnight at room temperature. After this incubation, the interior of the shells were scrubbed with sterile LBS to collect cells. The collected cells were washed, diluted, and plated as described above.

4. **Biogenic conjugation ("*in intestino*" conditions).** Donor and recipient cells were prepared as above. Healthy common quahogs (*Mercenaria mercenaria*) from the Marine Resources Center were individually washed in fresh seawater, and placed into a finger bowl with approximately 300 ml of seawater. 1 ml of donor and recipient were added (one a time; approximately five minutes between additions) to each finger bowl containing one clam, and filter feeding activity observed. Air was bubbled into each bowl for three hours. The clams were left overnight in the finger bowls into which seawater was continually dripped, continually flushing the small containers with fresh oxygenated seawater. After this incubation period, the clams were killed and O.1 ml of gut sample washed, , diluted, and plated as described above.

RESULTS:

1. Tn4351 transposes at a frequency too low to be useful for the gene transfer assay described.

Ideally, a plasmid transfer assay would be ideal for these investigations, as broad host range plasmids can be mobilized into host bacteria at frequencies approaching 10% (6). A lower frequency as seen in

the transpositional assay, coupled with possible host restriction systems, could lead to transconjugant numbers below the limits of detection. This would be particularly important under suboptimal conditions, as might occur under natural conditions.

E. coli strain EM24NR was conjugated with the pEP4341 *E. coli* donor strain, BW19851, under the conditions described in Materials and Methods. As EM24NR lacks the *pir* gene required to allow pEP4341 to replicate, the transferred plasmid is soon lost, allowing only Tn4351 transpositional events to be observed as tetracycline resistant exconjugants.

As seen in Table One, the frequencies with which tetracycline resistant transconjugants were obtained were low: on the order of 10^{-6} per recipient cell. This is in agreement with standard transpositional frequencies and work previously done with Tn4351 (N. Shoemaker, personal communication). These frequencies were less than ideal for the experiments described.

2. Tn4351 does not transpose into a marine *Vibrio* strain at appreciable frequencies.

The model inferred in this investigation is as follows: enteric bacteria containing mobile DNA elements would wash into marine environments as raw sewage. The question is, are such bacteria capable of transferring their genetic information into marine bacteria commonly found in such environments?

To that end, a brightly luminescent *Vibrio* like bacterium (strain L1) was used as a potential recipient in plasmid transfer or transpositional assays. The bacterium was made resistant to antibiotics by spontaneous mutation, as described in Materials and Methods.

L1NR (L1 resistant to both nalidixic acid and rifampicin) was used in conjugational experiments as described above. Table Two demonstrates that neither pEP4341 nor Tn4351 can be detected in this organism post conjugation. Either transfer of pEP4341 does not occur, or transposition of Tn4351 remains below the limits of detection in this system.

For this reason, further use of this marine bacterium as a recipient of transferred DNA was curtailed.

3. Transfer of pEP4341 between *E. coli* strains occurs at frequencies high enough to be useful for gene transfer assays.

Due to the results above, further investigations focused on the efficiency of plasmid transfer between *E. coli* strains under the conditions described in Materials and Methods. Since *E. coli* strain SY327 does possess the *pir* gene necessary for pEP4341 maintenance, it was expected that transfer frequencies would be significantly higher than observed for EM24NR.

Table Three shows that plasmid transfer into SY327 is quite efficient on standard LB plate spot mating assays, and therefore useful for the experiments described.

4. pEP4341 conjugates at high frequencies into recipient *E. coli* cells on solid marine medium.

Some plasmid transfer mechanisms are quite sensitive to ionic conditions, such as sodium chloride or magnesium (W. Metcalf, personal communication). As seen in Table Three, standard spot matings on ASW (which contains 75% seawater) result in transconjugants at high frequencies, little different from those observed from spot matings on LB. Clearly, the ionic composition of seawater poses no significant barrier to the conjugational gene transfer assay proposed.

5. pEP4341 conjugates at high frequencies into recipient *E. coli* cells following suspension in sterile seawater.

Table Three also demonstrates that pEP4341 can be conjugated efficiently into SY327 while suspended in filter sterilized seawater. This is an unusual result, as conventional wisdom (6) dictates that pair formation between donor and recipient cells is a vital precondition for efficient conjugation. It could be that microparticles (which passed through a 0.2 micron filter) could act as support for pair formation. Alternately, the cells themselves could act as nucleation sites for dissolved salts in the seawater, allow aggregates of cells to form.

Regardless of mechanism, such a result suggests the possibility of efficient transfer of genetic information almost immediately upon release of enteric bacteria into a marine environment, without the necessity of a

attaching to or colonizing a solid substrate. Since the scenario described is little different than some natural conditions, this result merits further detailed investigation.

6. pEP4341 conjugates at high frequencies into recipient *E. coli* cells on a common marine surface.

Table Three also demonstrates that efficient conjugation of pEP4341 into SY327 can occur when donor and recipient are mixed upon a surface commonly found in the marine environment: bivalve shells. However, the results seen with conjugation in the mock water column suggests a simpler explanation; the surface may not be a necessary condition for gene transfer. Further investigation is merited.

7. pEP4341 conjugates at detectable frequencies into recipient *E. coli* cells within the intestine of filter feeding marine bivalves.

Table Three demonstrates the most unusual finding of this investigation: that pEP4341 can be efficiently transferred to SY327 after donor and recipient *E. coli* strains are "fed" to filter feeding mollusks.

As only 0.1 ml of gut contents were sampled (see Materials and Methods), the overall numbers were low, but significant and not artifacts (example: when 0.1 ml of gut contents were diluted and plated, 3.36×10^4 SY327 cells were identified per milliliter of intestinal homogenate, and 94 SY327 / pEP4351 cells were identified per milliliter). The transconjugants obtained were indeed SY327 containing pEP4341 based on phenotypes and drug resistances. Further experiments using larger amounts of gut contents will be useful in refining the conjugational values obtained.

This important result is well worth following up most carefully.

DISCUSSION:

These experiments must be repeated with great care, in some detail. Still the preliminary data remain compelling.

In summary, it is clear that genetic material can be transferred among *E. coli* strains in diverse marine-like environments. This process can occur at moderately high frequencies (refer to Table Three) on agar plates, surfaces found commonly in marine environments (shells), within the water column, and within the intestinal tract of marine filter feeding bivalves.

The results with conjugation on surfaces and within the water column have been observed in some natural DNA-mediated transformation and to a lesser degree in freshwater conjugational systems (4, 5,16,17, 18). Still, the high frequencies reported make conjugation within the marine environment quite feasible as a method by which genetic information can spread efficiently among diverse species of bacteria.

The result obtained with conjugation in the water column is surprising, as pair formation would appear difficult to achieve under such conditions; small aggregations of cells in the water column due to microparticles or precipitates may circumvent this problem, as mentioned above.

The conjugational phenomenon within the water column may also be significant under natural conditions, as enteric bacteria are released into the marine environment in large quantities daily; it should be possible to monitor or evaluate this process *in vivo*. This result also casts doubt on many experiments necessitating a solid surface on which conjugation can occur (including one in this report measuring conjugal transfer on a solid surface). In such experiments, the observed conjugation may be occurring before the surface is actually colonized, while the cells are still suspended. A simple time-course study should clarify this issue.

Several reports of "biogenic" conjugation occurring within a metazoan (1, 2, 8, 11) exist in the literature, but none involve edible filter feeding mollusks. The possibility of gene transfer within the digestive tract of such bivalves is perhaps not surprising, given the large numbers of bacteria filtered from the surrounding environment (10, 12). Transconjugants within the bivalve gut might, after ingestion by humans, lead to further transfer within the human intestinal tract (14). This provides yet another route by which antibiotic resistance genes can spread into new species of bacteria.

Considering the numbers of raw filter feeding bivalves consumed world wide, the frequencies of conjugational plasmid transfer observed make this specific experiment of both theoretical and practical interest.

These results must be repeated and confirmed with a more appropriate assay of conjugational activity (as described above) as well as other bivalves of commercial importance, such as oysters.

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TABLE ONE: Gene Transfer Between *E. coli* strains (Transposition Assay)

Condition	Frequency (per recipient)
LB spot mating	4.30×10^{-6}
ASW spot mating	1.60×10^{-5}
Water column	1.90×10^{-6}
Shell surface	1.60×10^{-7}
Gut transfer	$<2.86 \times 10^{-3}$ (no colonies detected)

Note: "no colonies detected" refers to a lack of transconjugants; values are determined from the number of possible recipients.

TABLE TWO: Gene Transfer from *E. coli* into a Marine *Vibrio* Species (Transposition Assay)

Condition	Frequency (per recipient)
LB spot mating	$<5.58 \times 10^{-10}$ (no colonies detected)
ASW spot mating	$<1.47 \times 10^{-9}$ (no colonies detected)
Water column	$<4.98 \times 10^{-9}$ (no colonies detected)
Shell surface	$<1.52 \times 10^{-8}$ (no colonies detected)
Gut transfer	$<1.66 \times 10^{-5}$ (no colonies detected)

Note: "no colonies detected" refers to a lack of transconjugants; values are determined from the number of possible recipients.

TABLE THREE: Gene Transfer Between *E. coli* strains (Plasmid Transfer Assay)

Condition	Frequency (per recipient)
LB spot mating	6.90×10^{-3}
ASW spot mating	8.56×10^{-2}
Water column	2.53×10^{-3}
Shell surface	1.43×10^{-2}
Gut transfer	2.78×10^{-3}