

Report for 2001IA1241B: Effect of Swine Waste Effluent Field Application on Tn916 Content of Surface Waters

- Articles in Refereed Scientific Journals:
 - Haack, Bradley and Robert Andrews, 2000 Isolation of Tn916-like conjugal elements from swine lot effluent. Can J Microbio 46:542-549.

Report Follows:

Problem and Research Objectives:

Our previous work has shown that (i) Tn916 may be introduced in to the soil by application of manure containing wastes, (ii) that Tn916 mediated genetic exchange occurs in the soil, and (iii) that members of the normal soil microflora may receive Tn916. The experiments contained herein are designed to provide evidence that Tn916-containing enterococci appear in the surface waters after application of manure-containing wastes. The results will substantially "close the loop" by showing that fecal enterococci the water supply, which would then provide a mechanism by which these may be taken up by animals and humans. The strength of the current approach is that the analysis will depend on detection of specific DNA sequence responsible for gene mobilization as well as the presence of the specific antibiotic resistance gene.

To test the hypothesis, three lines of experimentation will be conducted

Objective 1

Examine effect of swine manure on the Tn916-containing fecal enterococci in surface water after waste application to nearby fields. Water samples from streams, ditches and drainage tiles will be examined. If the hypothesis is correct, one should observe that application substantially increases the Tn916 content in the enterococci found therein.

Objective 2

Determine if manure application in the field results in increased numbers of Tn916-encoded tetracycline resistant microbes in nearby surface waters. The content in these microbes will be determined in surface waters in the same sites as described in the previous objective. If the hypothesis is correct, application should increase the Tn916 content of these microbes.

Objective 3

Evaluate the *Sphaerotilus-Leptothrix* group of bacteria as an indicator of "genetic pollution" by antibiotic resistance genes. One of the requirements for monitoring "genetic pollution" will be the development of indicators that show that such events have occurred. Although the enterococci are important indicators in the short term, their survival in aquatic systems will be limited (2). Thus it is proposed to examine organisms that are more persistent in aquatic ecosystems for the presence of Tn916. Accordingly the members of the *Sphaerotilus-Leptothrix* group, particularly *Sphaerotilus* spp., will be examined for Tn916 content. If Tn916 were being passed through the water one would expect these organisms to acquire the genetic element after manure application.

Methodology:

Objective 1. The site selected for study was a field approximately 6 km north of Jewell Iowa. Water in drain tiles prior to manure application were sampled for the presence of enterococci, then after application, repeated samples were taken. Enterococcal isolation was done according the using selection of KF agar (5). Antibiotic resistance of these isolates was done according to the methods previously described (4).

Objective 2. The extraction of DNA from soils that is suitable for PCR amplification has been somewhat problematic. Currently we are using a method involving breakage of the cells in a Bead Beater followed by purification using HPT and gel filtration (3). DNA from pit wastes was extracted by using a modification of the method of Yeates et al (14).

Objective 3. The methods for isolation and growth of *Sphaerotilus* species are as described elsewhere in the literature (1, 6, 11). DNA was isolated from *Sphaerotilus* isolates by using the method of Mandel (7).

PCR methods. The PCR methods used herein were those described by Haack and Andrews (5) except that, in some experiments, the following primers were used:

tetM primers

Upper primer

GCGTACAAGCACAAACTCGT

Lower primer

CTCTAACCGAATCTCGTAAAT

ORF13 primers

Upper primer

GGGTACTTTTAGGGCTTAGT

Lower primer

GGCTGTCGCTGTAGGATAGAG

Principal Findings and Significance:

Objective 1. To begin this objective, it was of interest to determine the nature of the tetracycline resistance in fecal enterococci. Isolates of *Enterococcus faecalis* obtained from a swine farrowing house outflow were examined for genetic elements similar to Tn916. Of the enterococci isolated, 71% were resistant to tetracycline. Among the tetracycline-resistant enterococci isolated from the outflow samples, approximately 34% were able to transfer the tetracycline resistance phenotype to *Bacillus thuringiensis* in cross-genus matings. The frequencies of transfer for 10 random isolates were comparable to those for transfer of Tn916 from *E. faecalis* to *B. thuringiensis*. In addition, these elements were shown to mobilize plasmid pC194 between *Bacillus* species, as did Tn916. Southern blot and polymerase chain reaction (PCR) analysis showed these elements share extensive structural homology with Tn916. The selected conjugal elements were capable of transfer to a *Bacillus* recipient in a soil environment. When the swine waste was introduced into the soil, the tetracycline resistant fecal enterococci levels rose from essentially undetectable levels to approximately 4×10^4 and remained at this level for 4 weeks. After six months, including one winter, levels had decreased to 5×10^3 .

In samples from the field tiles, the patterns of tetracycline resistance in fecal enterococci was quite different. In the tiles, and in soil from the field, approximately 50% were resistant to tetracycline and of about 80% of these isolated appeared to contain Tn916, based on the ability to transfer the element to *Bacillus* recipients. Thus, it appears that Tn916 is more stable in the field than are other tetracycline resistance elements.

Based on these results, it was of interest to compare the fecal enterococci in the pit samples with those from field samples for their antibiotic resistances. For antibiotics are chosen for these studies are shown in Table 1.

Table 1. Percent of *Enterococcus faecalis* isolates resistant to various antibiotics.

Antibiotic	Percent resistant in pit samples ¹	Percent resistant in the field ¹
ampicillin	60	22
kanamycin	52	48
neomycin	46	60
tetracycline	72	50
vancomycin	66	62
streptomycin	32	38

¹Based on a sample size of 50 isolates obtained without antibiotic selection. These isolates were then examined for their resistance to the various antibiotics.

These data show that some antibiotic markers are more stable than others when the enterococci are in the soil. The basis for the variable instability under field conditions remains unclear.

Objective 2. To isolate DNA from swine pit samples a modification of the method of Picard was used (8). The method resulted in the isolation of DNA that was readily amplifiable using the ORF13 primers described in the methods section above. Figure 1 shows the amplified product from one such experiment.

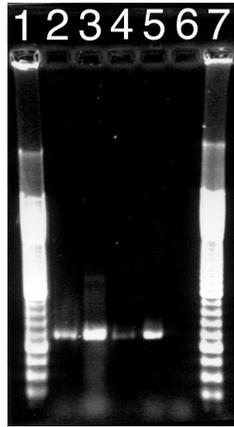


Figure 1. PCR products from DNA extracted from pit samples. Lane assignments, Lane 1, 100 bp ladder; Lane 2, positive control, Lane 3, undiluted extract, Lane 4; extract diluted 1:5; Lane 5, extract diluted 1:25; Lane 6, Negative Control, Lane 7, 100 bp ladder.

These data indicated that a non-antibiotic resistance-encoding region of *Tn916* abounds in the pit samples.

It was of further interest to examine this material for the presence of antibiotic resistance genes. In initial experiments the *tetM* primers described in the methods section of this report were used to amplify a fragment of DNA encoding a portion of the *tetM* gene, which exists in several forms in nature, including that found on *Tn916*. In these experiments, (data not shown) a fragment of DNA was readily amplified from the pit waste extract.

To further analyze these fragments, denaturing gel gradient electrophoresis (DGGE) was used. The methods for DGGE are described elsewhere (9, 10, 12, 13). The DGGE procedure allows one to examine the contents of a PCR reaction for the presence of different forms of very similar genes; the forms of the gene may differ in as few as 1 bp. Figure 2 shows the results from one such experiment.

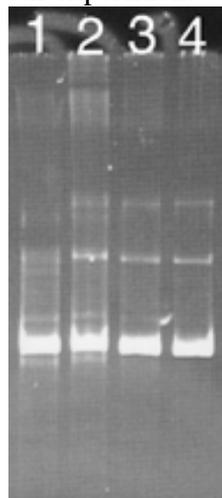


Figure 2. DGGE analysis of PCR product from pit waste. Lane assignments, ; Lane 1, positive control, Lane 2, undiluted extract, Lane 3; extract diluted 1:5; Lane 4, extract diluted 1:25.

In these experiments at least two unique bands are observed in PCR reactions from pit sample extracts suggesting that there are at least 3 forms of Tn916 conjugation gene in the pit sample wastes. Further characterization of these products is presently underway.

Objective 3. With regard to the third objective in the current proposal, we needed to perfect our techniques for *Sphaerotilus* isolation. Mat samples from the trickling filter were obtained from the Nevada, IA municipal sewage treatment plant. These isolates were brought to the laboratory and washed five times in sterile tap water. The mat was then broken up by using a Dounce homogenizer and diluted in sterile water. Samples from each dilution were plated on a medium modified from that of Stokes (11). The Stokes medium, which was designed to select for *S. natans* based on the low nutrient content of the medium, used 0.1% glucose and 0.1% tryptone in basal salts. In our hands, however, problems were encountered with the original Stokes medium. Many non-*S. natans* colonies appeared on the medium and we found that molds tended to overgrow the *S. natans* colonies that did come up. After several attempts we settled on a basal salts medium that contained no tryptone, glucose at 0.1%, 5 µg/L vitamin B₁₂, and 40 mg/L cyclohexamide to control mold growth.

When tetracycline (40 µg/ml) was included in the isolation medium, approximately 10% of the isolated appeared to be tetracycline resistant. DNA was extracted from 30 of these purified and used in PCR reactions using the primers of Haack and Andrews (5). Two of the samples exhibited a positive reaction, indicating the presence of a Tn916-like element. Thus the initial data suggests that Tn916 may exist in *S. natans* in a domestic sewage plant. With this information we have established that Tn916-like elements do appear in *S. natans*.

Literature Cited.

1. **Armbruster, E. H.** 1969. Improved technique for isolation and identification of *Sphaerotilus*. *Appl Microbiol* **17**(2):320-1.
2. **Borrego, J. J., and M. J. Figueras.** 1997. Microbiological quality of natural waters. *Microbiologia* **13**(4):413-26.
3. **Dojka, M. A., P. Hugenholtz, S. K. Haack, and N. R. Pace.** 1998. Microbial diversity in a hydrocarbon- and chlorinated-solvent- contaminated aquifer undergoing intrinsic bioremediation. *Appl Environ Microbiol* **64**(10):3869-77.
4. **Ferris, K. E., R. E. Andrews, Jr., C. O. Thoen, and B. O. Blackburn.** 1992. Plasmid profile analysis, phage typing, and antibiotic sensitivity of *Salmonella* dublin from clinical isolates in the United States. *Vet Microbiol* **32**(1):51-62.
5. **Haack, B. J., and R. E. Andrews.** 2000. Isolation of Tn916-like conjugal elements from swine lot effluent. *Can J Microbiol* **46**:542-549.
6. **Johnson, D. B., M. A. Ghauri, and M. F. Said.** 1992. Isolation and characterization of an acidophilic, heterotrophic bacterium capable of oxidizing ferrous iron. *Appl Environ Microbiol* **58**(5):1423-8.
7. **Mandel, M., A. Johnson, and J. L. Stokes.** 1966. Deoxyribonucleic acid base composition of *Sphaerotilus natans* and *Sphaerotilus discophorus*. *J Bacteriol* **91**(4):1657-8.

8. **Picard, C., C. Ponsonnet, E. Paget, X. Nesme, and P. Simonet.** 1992. Detection and enumeration of bacteria in soil by direct DNA extraction and polymerase chain reaction. *Appl Environ Microbiol* **58**(9):2717-22.
9. **Short, S. M., and C. A. Suttle.** 1999. Use of the polymerase chain reaction and denaturing gradient gel electrophoresis to study diversity in natural virus communities. *Hydrobiologia* . May **401**:19-32.
10. **Silvey, P., P. C. Pullammanappallil, L. Blackall, and P. Nichols.** 2000. Microbial ecology of the leach bed anaerobic digestion of unsorted municipal solid waste. *Water Science and Technology*. [print] **41**(3):9-16.
11. **Stokes, J. L.** 1954. Studies on the filamentous sheathed iron bacterium *Sphaerotilus natans*. *J Bacteriol* **67**:278-291.
12. **Straub, K. L., and B. E. Buchholz-Cleven.** 1998. Enumeration and detection of anaerobic ferrous iron-oxidizing, nitrate- reducing bacteria from diverse European sediments. *Appl Environ Microbiol* **64**(12):4846-56.
13. **Yang, C. H., and D. E. Crowley.** 2000. Rhizosphere microbial community structure in relation to root location and plant iron nutritional status. *Appl Environ Microbiol* **66**(1):345-51.
14. **Yeates, C., M. R. Gillings, A. D. Davison, N. Altavilla, and D. A. Veal.** 1997. PCR amplification of crude microbial DNA extracted from soil. *Lett Appl Microbiol* **25**(4):303-7.