PCR detection of Tn916-like elements in agricultural soil after the application of swine waste

Jay Howard King

Iowa State University

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PCR detection of Tn916-like elements in agricultural soil after the application of swine waste

by

Jay Howard King

A thesis submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of
MASTER OF SCIENCE

Major: Microbiology

Program of Study Committee:
Robert E. Andrews Jr. (Major Professor)
Alan Dispirito
Thomas Loynachan

Iowa State University
Ames, Iowa
2003

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Graduate College
Iowa State University

This is to certify that the master's thesis of

Jay Howard King

has met the thesis requirements of Iowa State University

Signatures have been redacted for privacy
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GENERAL INTRODUCTION

Producers have incorporated antibiotics into animal feed for disease prophylaxis and growth promotion since the 1950’s (58); annually in the United States, some $1.6 \times 10^6$ lbs of antibiotics are used in food animal production (19). Antibiotic resistance may be the most important problem in modern medicine (65). There is a growing concern that the agricultural use of antibiotics assists in the development and spread of antibiotic resistance in bacteria (1, 66, 122); acquisition of transposons or other mobile genetic elements may be partially responsible for this trend (48, 65, 126). Moreover, several reports suggest that the overall increase in antibiotic resistance observed in humans is linked to antibiotic use in food animal production (1, 3, 126). Transposons and other conjugative elements have been identified in association with methicillin-resistant *Staphylococcus aureus* (25, 84, 102), vancomycin resistant enterococci (9, 33, 55, 56) and antibiotic resistant *Streptococcus pneumonia* (33, 71, 91).

Previous studies in this laboratory have examined Tn916-mediated gene transfer in environmental settings. Haack et al. (39), for instance, demonstrated the conjugal transfer of Tn916 and Tn916 mobilization of the non-conjugal plasmid, pC194, between *Bacillus subtilis* and *Bacillus thuringiensis* in sterile and non-sterile soil microcosms. Subsequently, Haack and Andrews (38), using Southern hybridization and the polymerase chain reaction (PCR), with primers specific for OFR13 of Tn916, showed that 34% of *Enterococcus faecalis* isolates from swine manure contained Tn916-like elements that could be conjugally transferred to *B. subtilis* or *B. thuringiensis* in soil microcosms. This finding combined with the common practice of applying swine waste to agricultural soil led to the hypothesis tested
herein; that the application of swine waste to agricultural soils may facilitate the spread of antibiotic resistance among bacteria in the environment.

The goal of this research was two-fold. The first was to reproducibly extract DNA from soil that is amenable to PCR. The second was to utilize this DNA to detect the presence of Tn916-like elements in effluent-soil microcosms at various times post application.

**Thesis Organization.** This thesis consists of a general introduction followed by a literature review, materials and methods, results, discussion, acknowledgements and references. The master’s candidate, Jay H. King, is the senior author of this manuscript.
The Tn916 family of transposons. Several reports show that Tn916-like elements are widely dispersed in nature (12, 33, 35, 38, 72, 90, 91). In addition, Tn916 has been shown to transfer the tetracycline resistance determinant, tetM, between numerous Gram-positive and Gram-negative bacterial species (6, 34, 44, 54, 79-81, 89, 117). This mobile genetic element was originally identified in a dental isolate of E. faecalis and is found on plasmids such as the hemolysin plasmid, pAD1, or chromosomally located in the host genome (34). Tn916 contains the tetM gene that encodes a 72 kDa protein, which mediates resistance to tetracycline by protecting the translation initiation complex (10). Tn916 is the prototype of a broad family of related transposons (Table 1).

Work in this laboratory has focused on the molecular mechanisms of conjugative transfer of Tn916 and Tn916-mediated plasmid mobilization. Naglich et al. (80) reported Tn916-mobilization of co-resident non-conjugative plasmids, pC194 and pUB110, in matings between B. thuringiensis and B. subtilis. Moreover, Showsh et al. (108) provided evidence that the oriT of non-conjugal plasmids, such as pC194, are nicked by the same protein that nicks the oriT of Tn916 during conjugal transfer.

Tn916 structure and function. Flannigan et al. (32) sequenced Tn916 and identified 24 open reading frames spanning 18.3-kb in length (Fig 1). Located at the left end of Tn916 in Figure 1, ORF1 codes for a 8.1 kDa protein designated Xis-Tn, that has been shown to enhance excision of the transposon from the host DNA molecule (67), while ORF2 codes for the integrase protein (Int-Tn), which is required for both the excision and integration of Tn916 during conjugal transfer from the donor to the recipient DNA molecule (114). The
FIG. 1. Structure of Tn916. The oriT is located in the region between ORF20 and ORF21. ORF3 and ORF4 are located adjacent to Xis-Tn. Arrows represent the direction of transcription. Courtesy of R.E. Andrews.
Table 1. Tn916-like family of conjugative transposons

<table>
<thead>
<tr>
<th>Element</th>
<th>Size (kb)</th>
<th>Host Species</th>
<th>Resistance Determinant&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Clinical isolate</th>
<th>Composite Element&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Reference</th>
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<tr>
<td>CTnscr94</td>
<td>100.0</td>
<td><em>Salmonella senftenberg</em></td>
<td>scr</td>
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<td>(45)</td>
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<td>TnFOJ</td>
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<td>tetM</td>
<td></td>
<td></td>
<td>(86)</td>
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<td>Tn521</td>
<td>18.5</td>
<td><em>Streptococcus</em></td>
<td>ermB, tetM</td>
<td></td>
<td></td>
<td>(4, 92)</td>
</tr>
<tr>
<td>Tn916</td>
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<td><em>Enterococcus faecalis</em></td>
<td>tetM</td>
<td></td>
<td></td>
<td>(34)</td>
</tr>
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<td>Tn918</td>
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<td>Tn919</td>
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<td>tetM</td>
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<td></td>
<td>(31)</td>
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<td>Tn920</td>
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<td>(78)</td>
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<td>Tn925</td>
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<td>tetM</td>
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<td></td>
<td>(16)</td>
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<td>Tn1545</td>
<td>25.2</td>
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<td>ermAM, tetM, aph</td>
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<td>Tn1549</td>
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<td>vanB</td>
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<td>Tn3703</td>
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<td><em>Staphylococcus</em></td>
<td>tetM</td>
<td></td>
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<td>(62-64)</td>
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<td>Tn3704</td>
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<td><em>Streptococcus anginosus</em></td>
<td>ermB, tetM</td>
<td></td>
<td></td>
<td>(17)</td>
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<td>Tn3702</td>
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<td>tetM</td>
<td></td>
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<tr>
<td>Tn3872</td>
<td>23.5</td>
<td><em>Streptococcus defectivus</em></td>
<td>ermB, tetM</td>
<td></td>
<td></td>
<td>(72)</td>
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<td>Tn3951</td>
<td>67.0</td>
<td><em>Streptococcus</em></td>
<td>tetM</td>
<td></td>
<td></td>
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<td>Tn4399</td>
<td>9.9</td>
<td><em>Bacteroides fragalis</em></td>
<td>tetM</td>
<td></td>
<td></td>
<td>(43)</td>
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<tr>
<td>Tn4555</td>
<td>12.5</td>
<td><em>Bacteroides fragalis</em></td>
<td>cfxA</td>
<td></td>
<td></td>
<td>(111)</td>
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<td>Element</td>
<td>Size (kb)</td>
<td>Host Species</td>
<td>Resistance Determinant(^a)</td>
<td>Clinical isolate</td>
<td>Composite Element(^b)</td>
<td>Reference</td>
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<td>Tn5031</td>
<td>N.P</td>
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<td>tetM</td>
<td>+</td>
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<td>(33)</td>
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<td>Tn5032</td>
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<td>Tn5251</td>
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<td><em>Streptococcus pneumonia</em></td>
<td>tetM</td>
<td></td>
<td></td>
<td>(92)</td>
</tr>
<tr>
<td>Tn5252</td>
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<td><em>Streptococcus pneumonia</em></td>
<td>aph</td>
<td></td>
<td></td>
<td>(4)</td>
</tr>
<tr>
<td>Tn5253(^d)</td>
<td>65.5</td>
<td><em>Streptococcus pneumonia</em></td>
<td>cat, str, tetM</td>
<td>+</td>
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<td>Tn5276</td>
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<td><em>Lactococcus lactis</em></td>
<td>nis, scr</td>
<td></td>
<td></td>
<td>(95)</td>
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<td>Tn5381</td>
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<td><em>Enterococcus faecalis</em></td>
<td>tetM</td>
<td></td>
<td></td>
<td>(99)</td>
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<td>Tn5382</td>
<td>27.0</td>
<td><em>Enterococcus faecium</em></td>
<td>vanB, ppb5</td>
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<td>Tn5384</td>
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<td><em>Enterococcus faecalis</em></td>
<td>erm, tetM</td>
<td>+</td>
<td></td>
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<td>Tn5385</td>
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<td><em>Enterococcus faecalis</em></td>
<td>ermB, tetM</td>
<td>+</td>
<td></td>
<td>(98)</td>
</tr>
<tr>
<td>Tn5397</td>
<td>20.7</td>
<td><em>Clostridium difficile</em></td>
<td>tetM</td>
<td></td>
<td></td>
<td>(77)</td>
</tr>
<tr>
<td>Tn5398</td>
<td>9.6</td>
<td><em>Clostridium difficile</em></td>
<td>ermB</td>
<td></td>
<td></td>
<td>(30)</td>
</tr>
</tbody>
</table>

\(^a\) Conferring genetic resistance to: tetM-tetracycline, erm-erythromycin, van-vancomycin, str-streptomycin; ppb-penicillin, aph-kanomycin, cat-chloramphenicol; nis and scr-carries genes coding for nisin production and sucrose fermentation respectively.

\(^b\) Element is comprised of 1 or more transposable elements.

\(^c\) N.P. indicating transposon size is not published.

\(^d\) Element composed of the conjugal transposon Tn917 integrated into a Tn916-like element.

\(^e\) Table does not include individual plasmids or bacterial genomes that share sequence similarity with Tn916-like elements.
tetM gene is located in the region corresponding to ORF11, with a possible promoter for this determinant located within ORF12 (115). TnPhoA (69) insertions into ORF13 suggest this region codes for a membrane targeted protein (Loe and Andrews unpublished).

Scott et al. (106) identified a covalently-closed circular intermediate of Tn916 that was capable of transforming protoplast of Bacillus subtilis. According to the model; while in the donor cell, a protein nicks the oriT of Tn916, which releases a single stranded copy of the transposon that ligates at its termini, forming a circular intermediate that subsequently transfers to the recipient during conjugation (105). Once in the donor cell, integration of the circular intermediate is thought to occur as the reverse of the excision process, wherein, staggered cuts are made simultaneously on both sides of the circular intermediate and the recipient DNA molecule, followed by the integration of a single stranded copy of Tn916 into the recipient DNA (11). Moreover, Tn916 transfer into the recipient DNA preferentially occurs at AT rich sequences (51, 105, 118).

The presence of tetracycline enhances both conjugal transfer and plasmid mobilization of Tn916. For example, Showsh et al. (109) showed that the conjugal transfer of Tn916 between E. faecalis and B. thuringiensis was enhanced nearly 15-fold; and plasmid mobilization enhanced nearly 120-fold, when cells were pre-incubated with tetracycline prior to filter mating. Similarly, Mangenelli et al. (68) reported a 50-fold increase in both circular intermediate formation and conjugation frequency with tetracycline in the growth medium. Furthermore, Doucet-Populaire et al. (27) reported a 10-fold increase in the in vivo conjugal transfer of the Tn916-like transposon, Tn1545, between E. faecalis and L. monocytogenes in the digestive tracts of mice in the presence of tetracycline. As these studies indicate,
tetracycline not only enhances transfer of Tn916-like elements but stimulates plasmid mobilization both *in vitro* and *in vivo*.

Research in our laboratory has focused on the transfer of Tn916-like elements from fecal bacterial populations to indigenous soil microflora after application of swine wastes to soil. Four questions relevant to this issue are reviewed herein.

Do mobile genetic elements mediate gene transfer in nature?

Do mobile genetic elements mediate gene transfer in soil?

Do mobile genetic elements mediate gene transfer in manure?

Do mobile genetic elements mediate gene transfer in manure-applied soil?

**Gene transfer of Tn916 and Tn916-like elements in nature.** The evidence is clear, Tn916-mediated gene transfer has been demonstrated in several different habitats. For example, Roberts *et al.* (101) reported transfer of the Tn916-like element, Tn5397, between *B. subtilis* and *Streptococcus acidominimus* in a dental plaque; and in a recent report (100), the transfer of other Tn916-like elements between *E. faecalis* and various *Streptococcus* species in dental plaques. Moreover, Shoemaker *et al.* (107) showed that the Tn916-like transposon, CTnDOT, transfers the *tetM* determinant between *Bacteroides* species in the human colon. In addition, Doucet-Populaire *et al.* (27), as previously mentioned, reported the *in vivo* transfer of the Tn916-like element, Tn1545, from *E. faecalis* to *L. monocytogenes* in the digestive tracts of mice. As these studies indicate, the broad host range of Tn916 is not only an *in vitro* phenomena, but one that extends into natural settings.

**Horizontal gene transfer in soil microcosms.** Soil components such as clay have been shown to provide a surface for the cell to cell contact that is required for bacterial conjugation (123). And as the following reports suggest, may provide niche for conjugal
gene transfer between bacteria. The plasmid-mediated transfer of antibiotic resistance genes in soil has been observed in several studies. Rafii et al. (94) for example, reported both conjugative transfer and co-mobilization of nonconjugal plasmids, such as plJ303, between indigenous *Streptomyces* in soil microcosms. Moreover, Digiovanni et al. (26) demonstrated transfer of the mercury resistance determinant, *tfbD*, from *Alcaligenes eutrophus* strain JMP134 (containing an IncP type plasmid) to an indigenous soil bacterium in non-sterile soil microcosms. *Pseudomonas glathei* and *Burkholderia caryophyllii* were identified as the indigenous recipient species. Similarly, Newby et al. (82), reported the transfer of pJP4 from *E. coli* to the indigenous soil bacterium, *Bulkholderia*, in non-sterile soil microcosms. Thus, lateral gene transfer of drug resistance plasmids has been observed in native and sterile soil microcosms and provides a mechanism for the recipient cell to survive in the presence of antibiotics.

Interestingly, soil invertebrates are vectors of gene transfer. Danne et al. (23) for instance, reported transfer of pJP4 in non-sterile soil microcosms, containing earthworms, between spatially separated donor *A. eutrophus* and recipient *P. fluorescens*. The earthworms were evidently vectors for transporting the donor and recipient species because transconjugants were not detected in the absence of earthworms.

Haack et al. (39) demonstrated (i) conjugal transfer of Tn916 between *B. subtilis* and *B. thuringiensis* at a frequency of $10^{-5}$ transconjugants / donor and (ii) Tn916 mobilization of the non-conjugal plasmid pC194 between these strains at a frequency of $10^{-6}$ transconjugants / donor in sterile and non-sterile soil microcosms. The presence of the indigenous soil microflora seemed to have no effect on gene transfer, because Tn916 conjugation was observed at comparable frequencies in both autoclaved and native soil microcosms. Studies
such as these provide insights into gene transfer in soil, and demonstrate that indigenous soil microbial populations can be the recipients of such gene transfer events. The plasmid-mediated acquisition of antibiotic resistance genes by indigenous soil species are significant because these populations could serve as a reservoir of antibiotic resistance genes and facilitate their spread to other microbial populations in the environment.

**Antimicrobials in livestock production and antibiotic resistance dissemination.**

Evidence shows that administration of antibiotics to livestock correlates with resistance levels in enteric bacteria populations. Dawson *et al.* (24), for example, compared antibiotic resistance levels in anaerobes and coliforms from swine herds exposed to high levels chlorotetraacycline in the diet and compared these to herds with low exposure to the antibiotic. As expected, higher levels of chlorotetraacycline resistant anaerobes were reported in high level exposure herds (81%), compared to resistance levels in low level exposure herds (27%); similar observations were made regarding coliforms (48% verses 21%). Other studies have shown that antibiotic resistant fecal bacterial populations remain elevated after the withdraw of the antibiotics. Langlois *et al.*, (60), for instance, showed that tetracycline resistance among fecal coliforms from swine waste declined to 42% of peak, ten years after antibiotic withdraw.

Studies have shown that antibiotic resistance levels correlate with the use of growth promoting agents. In Sweden, for example, growth-promoting antimicrobials were banned in 1986 whereas in the Netherlands, these agents were not banned. Van Den Bogaard *et al.* (121) compared antibiotic resistance levels in *E. coli* and fecal enterococci from these regions and reported a significantly higher prevalence of resistance to oxytetracycline and amoxycillin in the Netherlands. Other studies have linked the use of growth promoters in
animal feed with the presence of transferable plasmids and transposons. Christie and Dunny (15), for instance, found that feeding tylosin to swine herds selected for plasmid mediated antibiotic resistance in fecal enterococci. Moreover, an association between the use avoparicin, a growth promoting agent, and an increase in transposon-mediated vancomycin and teicoplanin resistance among *E. faecium* was observed by Bager *et al.* (5); vancomycin resistant *E. faecium* was isolated from 11 of 12 farms where avoparcin was used. In contrast, vancomycin resistant *E. faecium* was isolated in only 2 out of 12 farms were avoparcin was not in use. Furthermore, the vancomycin resistant *E. faecium* isolates contained a *vanA* determinant on the non-conjugative transposon Tn1546. Although non-conjugative, Tn1546 is typically found in association with other mobile genetic elements. These studies suggest that conjugative plasmids and transposons play a role in the dissemination of antibiotic resistance determinants and may increase the threat resulting from drug resistance.

**Evidence of antibiotic resistant transfer between animal and human.** Early studies correlated antimicrobial use in agriculture with an increase in the incidence of antibiotic resistance among humans. In a seminal study, Levy *et al.* (66) compared tetracycline resistance levels in bacterial isolates farm animals and farm workers before and after the introduction of tetracycline; after 6 months, 31% of fecal coliforms from the farm workers contained high levels of plasmid-mediated tetracycline resistance. In a similar study, Hummel *et al.* (49) investigated the effects of incorporating nourseothricin (an antibiotic fed primarily to swine) in animal feed on a test farm. Over a two-year period, the researchers tested farm animals, farm animal handlers and people from the surrounding community for the presence of *E. coli* plasmids conferring resistance to this antibiotic. In this study, 33% of *E. coli* isolated from the feces of animals fed the antibiotic, and 17% of *E. coli* from the feces
of farm workers, contained high levels of plasmid borne-resistance to nourseothricin, suggesting that antibiotic resistant \textit{E. coli} (carrying R plasmids) from farm animals were able to colonize farm workers in close association.

Recent studies provide evidence that links antimicrobial resistance with conjugative transposons. For instance, van den Bogaard \textit{et al.} (120), working with vancomycin resistant enterococci, found identical pulsed field gel electrophoresis patterns in the \textit{vanA} determinant (located on Tn1546) from \textit{E. faecium} strains isolated from the feces of turkeys fed avoparcin (a glycopeptide antibiotic similar to vancomycin) and from turkey farmers with direct contact to the animal. Moreover, Tn1546 has been identified in vancomycin resistant enterococci of both human and animal origin from the same geographic region (52, 53, 55, 103, 125). Studies such as these suggest an initial horizontal acquisition of resistance genes by fecal enterococci followed by the clonal dissemination of resistance determinants (while in the animal), and the subsequent colonization of humans in close contact. Research by Hanarahan \textit{et al.} (41), working with the closely related \textit{vanB} determinant, supports this hypothesis. The researchers isolated vancomycin resistant enterococci (containing the \textit{vanB} and \textit{pbp5} determinants located on the conjugative transposon Tn5382) in enterococci from geographically separate locations in the US, indicating that drug resistant enterococci of animal origin (carrying the \textit{vanB} determinants) were able to colonize humans.

\textbf{Persistence of antibiotic residues in manure and manure-applied soils.} Because picogram concentrations of tetracycline stimulate conjugative transfer of Tn916 \textit{in vivo} (109), the presence of this antibiotic in swine waste may increase Tn916 transfer in the field soil following manure application. In a recent study, Hansen \textit{et al.} (42) used a novel whole cell bacterial biosensor and found that chlorotetracycline persists in manure for at least 30
days. Moreover, they showed that tetracycline resistance levels in coliforms remained elevated for a 30-day period following manure incorporation. In addition, Hamscher et al. (40) reported that tetracycline residues persist in soil at least 12 months after the application of liquid manure to farm soil; tetracycline not only remained detectable in the field from one application to the next, but accumulated after successive applications. Thus, chlorotetracycline and tetracycline persist in soil and manure, and may stimulate gene transfer in both.

**Bacterial survival in manure and manure-applied soils.** If gene transfer were to occur from bacteria released in animal wastes to indigenous soil bacterial populations, then an important question would be “How long does the bacterial population in waste remain viable after its addition to soil?” Several studies have attempted to answer this question. Lau and Ingham, for example, showed that *E. coli* and fecal enterococci survive at least 133 days after manure incorporation into soil (61). Moreover, Kearney et al. (57) inoculated several bacterial species into cattle manure slurry in soil and found culturable *Salmonella typhimurium, Yersinia enterocolitica, L. monocytogenes* and *Campylobacter jejuni* 112 days post manure application. Work in this laboratory by Haack and Andrews (38) showed that numbers of tetracycline resistant fecal enterococci decreased by about one order of magnitude 120 days after manure incorporation into soil. Therefore, fecal enterococci, coliforms, and a number of allochthonous microbes found in manure remain viable after the incorporation of manure into soil, and increase the opportunity of conjugative gene transfer therein.

**Gene transfer in animal waste, human waste and manure-applied soil.** Swine manure applied to soil introduces dramatic changes in the microbial community structure and
provides a microhabitat conducive to gene transfer. Gotz et al. (37), for example, demonstrated transfer of the nonconjugal plasmid pIE723 between inoculated E. coli and Pseudomonas pituda strains in manure-treated soil. In this study, pIE723 transfer was not observed in E. coli strains lacking a conjugative IncPα plasmid. The observation most germane to the current discussion, however, was that frequency of plasmid transfer increased 1000-fold, following the application of swine manure to soil.

Several reports provide evidence that Tn916-mediated gene transfer occurs in soil. For example, Agerso et al. (2) reported that 3.4% of Bacillus cereus (an allochthonous soil microbe) isolates from swine manure contained the tetM determinant in association with Tn916. Because the tetM determinant was transferred to E. faecalis under laboratory conditions, the evidence suggests that the drug resistance was associated with Tn916-like elements. In addition, Marcinek et al. (70) reported transfer of Tn916 at extrapolated frequencies of at 10^-8 transconjugants/donor between different E. faecalis strains in activated sludge basins. And recently, Chee-Sanford et al. (13) identified tetM determinants in both waste water isolates and in an indigenous soil bacterium (Bosea spp) located downstream from the swine production facilities. Studies such as these demonstrate in situ conjugal transfer of Tn916-like elements in these habitats, and provided the rationale for this study.

In the study leading to the research presented here, Haack and Andrews (39) monitored tetracycline resistant fecal enterococci in swine manure, and found that 71% of fecal enterococci were resistant to tetracycline. Of these, 34% where shown to contain Tn916-like elements, as determined by Southern hybridization and PCR. Isolates that appeared to contain Tn916-like elements were shown to conjugally transfer Tn916 to B. subtilis and B.
*thuringiensis* subsp *israelensis* in filter matings. In continuation of this research, the goal this study was to use PCR for the detection of Tn916-like elements in effluent treated soil microcosms.

**DNA extraction from soil.** To analyze bacterial genes in soil using either Southern hybridization or PCR, the cells must first be lysed. In the indirect method, originally developed by Faegri et al. (29), microbial cells are separated from the soil matrix in a Warring blender, then centrifuged to separate the bacterial fraction from the fungal mycelia and higher weight soil and sand particles. After cell separation, the DNA is extracted from bacterial cells and molecular methods are then used to characterize specific gene sequences. In the direct method of DNA extraction, developed by Ogram (83), the bacterial cells are lysed directly in the soil matrix by high speed homogenization with glass-beads. Following cell lysis, sand particles and cellular debris are removed by centrifugation. After which, the DNA is extracted. Since these early studies, several variations of the direct lysis method have been described for the physical disruption of microbes in soil including; freeze-thaw lysis (119), sonication (87), chemical /enzymatic lysis (116). In terms of detection sensitivities, the evidence suggests that the direct DNA extraction method is superior to the indirect extraction method. For instance, Holben et al. (46) extracted cells (indirectly), purified DNA and used Southern hybridization with a probe specific for the *nptII* to detect *Bradyrhizobium japonicum* in soil. In this study, DNA signal was observed in soils containing approximately $10^4$ CFU/ml. In contrast, Ogram et al. (83), using the direct DNA extraction method from soil and Southern Hybridization (with a probe specific for the *nif* gene) to detect this target sequence, obtained DNA signal from soil containing $10^3$ CFU/ml.
Soil organic matter consists of humin, humic acid and fulvic acid. Humin is the insoluble fraction of soil organic matter that cannot be extracted with a dilute alkali solution, while humic and fulvic acids are soluble in dilute alkali. In addition, humic acids precipitate in acidic solutions while fulvic acids remain soluble in acidic solutions. Humic acids are polymerized macromolecular structures consisting of carboxylic acids in association with both aromatic and phenolic rings interspersed with amino acid residues, polysaccharide and peptides moieties with molecular weights ranging from 100,000 to 300,000. Fulvic acids are highly oxidized aromatic compounds that contain a high proportion of polysaccharides with molecular weights ranging between 1,000-10,000. When co-extracted with DNA from soil, these compounds interfere with molecular techniques such as PCR (87, 116, 127), restriction enzyme analysis (97) and Southern hybridization (113). Thus, removal of these compounds during DNA extraction is paramount. Various combinations of chromatography including gel filtration, reverse phase and ion-exchange have been used to remove soil derived compounds from soil DNA prior to PCR or Southern Hybridization.

Hydroxylapatite was one of the first compounds used to effectively purify DNA extracts from soil (83). In this form of adsorption chromatography, the positively charged calcium ions in the stationary phase bind to negatively charged phosphate groups on the polynucleotide backbone of DNA at low salt concentrations (104). At high salt concentrations [typically ≥ 0.36 M sodium phosphate (pH 6.8)], double stranded DNA is eluted (104) and thus, separated from the humic acids, which are retained in the column. Ogram et al. (83), using a direct DNA extraction method, incorporated hydroxylapatite (in combination with 0.5 M potassium acetate) for soil DNA purification, and successfully removed soil derived compounds from the DNA extracts. In a similar study, Steffan et al. (113) used the
direct DNA extraction method and reported that hydroxylapatite columns effectively removed PCR inhibitors from soil DNA extracts; unfortunately its use resulted in a substantial loss of DNA. Purdy et al. (93) used a direct DNA extraction method in combination with hydroxylapatite column chromatography for purification and obtained high molecular weight DNA (>10 kb) from soil that was amendable to both PCR and Southern Hybridization. Thus, the use of hydroxylapatite column chromatography can effectively remove inhibiting substances from soil DNA extracts.

Another compound used to purify soil DNA extracts is polyvinylpolypyrrolidone. The carbonyl groups of this polyamide form hydrogen bonds with phenolic groups from humic acids found in crude soil extracts, thus, separating them from soil DNA (28). Holben et al. (46), using a modified indirect extraction method, found that the inclusion of polyvinylpolypyrrolidone in the purification procedure effectively removed humic substances prior to Southern hybridization. Moreover, Zhou et al. (128) compared soil DNA extracts purified with and without polyvinylpolypyrrolidone and reported its use substantially improved the DNA purity. However, the result was a decrease in DNA yield. Thus, polyvinylpolypyrrolidone is effective in the removal of inhibitors from DNA extracted from soil.

Another method of obtaining pure DNA from soil, is with size exclusion or gel-filtration chromatography. Sephadex resins are commonly used for this purpose, and are composed of cross-linked polymers of dextran, whose spherical beads contain pores that allow for the separation molecules based on size. In these columns, separation is achieved because the larger DNA molecules (MW typically exceeding > 10^9) elute in the void volume of the column, while smaller contaminating substances are retarded and slowed in the bead matrix. In previous studies, gel filtration resins such Sephadex G-75 have been used to desalt soil
DNA fractions from hydroxylapatite columns (93) and also used in combination with polyvinylpolypyrrolidone chromatography to purify soil DNA (22). In addition, resins with larger pore sizes such as Sephadex G-200 have been used alone (73) or in combination with; Bio-Gel P6 (119) or in combination polyvinylpolypyrrolidone column chromatography (59), to successfully purify soil DNA, thus, increasing the detection sensitivity using molecular methods. These studies and others provided the basis for incorporating gel filtration chromatography in these experiments.

To purify DNA from soils with high organic matter or high clay content, researchers have relied on silica based purification methods. Boom et al. (8) originally demonstrated DNA and RNA purification from human serum using DNA binding to silica or glass beads. Also called glassmilk purification; this form of reverse phase chromatography relies on the principal that the negatively charged phosphate backbone of the DNA molecule binds to the surface of glass beads in presence of the high chaotrophic salt (typically 6.0 M guanidium thiocyanate). Apparently, this occurs through the creation of a cation or salt or bridge between the oxygen atoms on the bead surface and the DNA backbone. The resin is sold under proprietary names such as Geneclean ® (Wizard, Promega, Madison WI), and Fast DNA spin sample kit ® (For soil; Bio 101, Lajolla, CA). Several investigators have used silica-based methods to purify DNA from soil. For instance, Zhou et al. (128) purified soil DNA with a silica-based resin (Wizard PCR Preps purification resin ®, Promega) and obtained PCR signal from the amplicon assayed. This and similar reports of successful soil DNA purification using silica based methods (8, 76, 110) provide the basis for using this methodology in these experiments.
Detection of bacterial genes in soil. Previous studies have shown that PCR is more sensitive than Southern hybridization and conventional culturing in detecting bacterial gene sequences from soil. Steffan et al. (112), for instance, using the direct DNA lysis method from soil, used PCR to successfully monitor the fate of genetically engineered micro-organisms released into soil, and obtained sensitivities 10-fold greater than those using Southern Hybridization. Moreover, Recorbet et al. (96), monitoring the survival of E. coli in soil, used Most Probable Number-PCR with DNA extracted from soil and detected signal from 16S rDNA from introduced E. coli after 70 days. In contrast, the detection level of E. coli by plate counting in this study was $10^2$ CFU/g $^{-1}$. Moreover, seeded E. coli could not be cultured beyond 15 days from soil microcosms. Findings such as these provided the rationale for using PCR in these experiments.

For our research, a combination of the proceeding methods was used to obtain DNA from soil that was amendable to PCR. In these experiments, a modified direct method of Chelius et al. (14) was used to extract DNA from soil. And to purify nucleic acids from soil, we used the method of Cullen et al. (22) with modifications. An additional stage of DNA purification was needed for the soil used in this study, thus, we relied on a proprietary soil DNA binding matrix. To detect Tn916-like sequences from soil, an optimized commercial PCR kit was used.
MATERIALS AND METHODS

*Enterococcus faecalis growth conditions.* *E. faecalis* CG110, containing one copy of 
Tn916 integrated into the genome (36), was used as a positive control in all procedures. 
Bacterial cells were grown overnight at 37°C on a rotary shaker at 150 rpm in Brain Heart 
Infusion (BHI, Difco Laboratories, Detroit, MI) supplemented with 20 µg/ml tetracycline 
(Sigma-Aldrich, St. Louis, MO). Solid media was prepared by adding 1.5% (w/v) agar 
(Difco) supplemented with 40 µg/ml tetracycline. Growth of *E. faecalis* was monitored by 
measuring absorbance at 600 nm in 30 min intervals. Cell numbers were confirmed by serial 
dilution and plating on BHI containing 20 µg/ml tetracycline. *E. faecalis* CG110 cells were 
grown to an O.D of 0.4 (approximately 10^8 CFU/ml) prior to DNA extraction. Tetracycline 
resistant plate counts from effluent-soil microcosms were performed at 37°C for 24 h on 
plate count agar (PCA, Difco) supplemented with 40 µg/ml tetracycline (Sigma-Aldrich) and 
reported in CFU/ml.

*Soil characteristics.* The soil described by Haack *et al.* (39) was used in all experiments, 
and was a Dickinson aeolian sand (coarse-loamy, mixed, mesic Typic Hapludolls) collected 
from 20-30 cm below the surface of a farm drainage ditch located 3.5 mi SE of Ames, IA. 
The average pH of the soil was 5.6 and had an organic matter content of 1.8%. Soil texture 
properties were as follows: 95.5% sand, 1.2% course silt, 1.3% fine silt and 4.0% clay. The 
soil was sieved through a steel screen (1-mm mesh) to remove plant material and stored at 
room temperature until use.

*Swine lot effluent.* Effluent samples were obtained from an above ground waste storage 
container located at Iowa State University Swine Nursery. Prior to these experiments, this
facility had no history of antimicrobial use for growth enhancement; intermittent therapeutic use of antibiotics was assumed.

**Soil DNA extraction.** *E. faecalis* CG110 (approximately $10^8$ CFU/ml) was serially diluted into 9-ml dilution blanks of 0.85 % saline solution. A 100-g soil sample was inoculated with 9 ml serial dilution blanks containing: $10^3$, $10^2$, and $10^1$ CFU/ml. The mock inoculation was conducted using a sterile saline blank. The cells were gently mixed into the soil using a sterile spatula then incubated at room temperature for 30 min. The DNA was extracted using a Beadbeater® (BioSpec Products Inc., Bartlesville, OK), and performed as described by Chelius *et al.* (14), with modifications.

Prior to DNA extraction; 19 g of 0.1 mm glassbeads (BioSpec Products Inc), 6 ml of bead-beating buffer (10 mM Tris-HCl-150 mM NaCl-100 mM EDTA- 4% SDS, pH 8.0) and 10 g of soil were added to a 32-ml polycarbonate bead-beater chamber. The chamber was sealed then placed inside of a 350-ml bead-beater ice jacket chamber (BioSpec Products Inc.) containing equal weights of ice and water. This apparatus was secured to the bead-beater and the samples were homogenized at full speed in 15 sec intervals; with chilling on ice for 1 min between intervals. All transfers were made using the large bore end of a 5-ml glass pipette. The sample slurry was transferred to a 150-ml Corex® centrifuge bottle, and the bead-beater chamber was washed with 3 ml of 10 mM Tris-HCl (pH 8.0); the washate was transferred to the Corex bottle. Glassbeads, soil particles and cell debris were pelleted by centrifugation at 6,100 x g for 8 min at 4°C (Beckman J2-21 centrifuge; JA-10 rotor). The supernatant was transferred to a 50-ml conical bottom centrifuge tube on ice. Pellets were washed and resuspended in 4 ml bead-beating wash buffer (10 mM Tris-HCl, pH 8.0), and centrifuged as above. The supernatant was added to a new conical bottom centrifuge tube. Acid-washed
polyvinylpolypyrrolidone (PVPP; Sigma-Aldrich, St. Louis, MO) was prepared by the method of Holben et al. (46) and added to the crude DNA extract. Two grams of acid-washed (finely ground) polyvinylpolypyrrolidone was added to the pooled supernatants, and incubated on ice with agitation using a orbital water bath (Sci-Era; Bellco Biotechnology, Vineland, NJ) at half speed for 30 min. Samples were centrifuged at 3,000 x g on a bench-top centrifuge (Beckman TJ-6 centrifuge; TA-10 rotor) for 5 min at room temperature. The supernatant was transferred to a new tube. The polyvinylpolypyrrolidone pellets were washed with 5 ml of 10 mM Tris-HCl, pH 8.0 by vortexing on high speed for 1 min, and centrifuged as above. The washate was recovered and pooled with the first supernatant. An equal volume of 10 mM Tris- HCl (pH 7.0)-saturated phenol was added to pooled samples in a sterile 150-ml Corex centrifuge bottle. The samples were vortexed at high speed for 3 min, then centrifuged at 12,000 x g at 4°C (Beckman J2-21 centrifuge; JA-10 rotor) for 15 min. The brown colored aqueous phase was transferred to a new centrifuge bottle and extracted with an equal volume of chloroform-isoamyl alcohol (24:1), then centrifuged as above. Nucleic acids were precipitated with two volumes of 95% ethanol, 2.5 M ammonium acetate (final concentration) and 20 µg/ml (final concentration) of purified Oyster Glycogen Type VII (Sigma) for 16 h at -20°C. The following day, the DNA was pelleted by centrifugation at 14,000 x g (Beckman J2-21 centrifuge, JA-10 rotor) for 15 min at 4°C. The DNA pellet was resuspended in 0.5 ml of 10 mM sodium phosphate buffer (pH 6.8) at 45°C for 10 min, and either used directly for purification or stored at −20°C.

The Crude DNA extract was purified using hydroxylapatite column chromatography (HTP, Fast Flow; Calbiochem San Diego, CA). The resin was prepared by suspending 30 g of solid hydroxylapatite in 150 ml of 10 mM sodium phosphate buffer (pH 6.8). The mixture
was autoclaved for 15 min at 121°C. Hydroxylapatite columns were assembled using a 5-ml syringe (plugged at the bottom with glasswool and fitted with a luer-lock to control liquid flow). Hydroxylapatite was resuspended by shaking then added to the syringe barrel until a packed volume of 5 ml was obtained. The columns were washed with 3 column volumes of 10 mM sodium phosphate buffer (pH 6.8), which were incubated at 45°C prior to use; then added to the columns which were assembled and ran at room temperature. Frozen DNA samples (0.5 ml) from the extraction step were incubated at 45°C for 30 min, and resuspended by vortexing at low speed for 30 sec, then loaded onto the top of a 5-ml hydroxylapatite column. The DNA samples were allowed to enter the column by gravity flow. Crude DNA samples were washed with 10 ml of 10 mM sodium phosphate buffer (pH 6.8). RNA was eluted from the columns by adding 10 ml of 140 mM sodium phosphate buffer (pH 6.8). DNA was eluted from the column with 475 mM sodium phosphate buffer (pH 6.8) into 0.5 ml fractions. The DNA content of each fraction was assayed using fluorescence by agarose gel electrophoresis (see gel electrophoresis of DNA extracts). Fractions were incubated at 45°C prior to isobutanol extraction. To confirm hydroxylapatite columns were not overloaded with soil DNA, 140 mM sodium phosphate wash fractions were pooled and nucleic acids were precipitated with 2 vol of 95% ethanol and 2.5 M ammonium acetate and incubated at -80°C for 30 min.

Fractions containing DNA were pooled and concentrated using isobutanol. Briefly, an equal volume of isobutanol was added to the fractions, vortexed for 20 sec then centrifuged at 14,000 x g (Eppendorf centrifuge 5415) for 20 sec at room temperature. The upper organic phase was discarded and the lower aqueous phase (containing the DNA) was subject
to further extraction. This step was repeated until the final volume of each fraction was approximately 100 µl (104).

Sephadex G-75 (Sigma) was prepared by suspending 7.5 g of powered Sephadex G-75 (Sigma) into 150 ml of distilled water. To remove fines, the suspension was heated to 70°C in a water bath and allowed to settle for 2 h. This step was repeated until fines were no longer observed. The matrix was resuspended in 150 ml of 10 mM Tris-HCl (pH 8.0). This mixture was autoclaved at 121°C for 15 min, and allowed to cool at room temperature overnight. Sephadex G-75 columns were assembled using a 25-ml glass pipette (cut in half), and plugged at the bottom with glass wool. A Tygon® hose was secured to the syringe barrel with a clamp to control liquid flow.

Isobutanol-condensed fractions were pooled to a total volume of 0.5 ml, then loaded onto a 7-ml Sephadex G-75 column, and allowed to enter the matrix by gravity flow. Elution was performed with a buffer containing 10 mM Tris-HCl (pH 8.0), which was slowly added to the top of the column, and continued until twelve - 0.5 ml fractions, were collected. The DNA content of each fraction was assayed as described for hydroxylapatite columns. Fractions containing DNA were pooled and precipitated with 2 vol 95% ethanol and 2.5 M ammonium acetate, then incubated at -80°C for 30 min. The DNA pellet was washed with 70% ethanol and centrifuged as above. The DNA pellets were resuspended in 1.2 ml of distilled water.

The DNA samples were further purified using a DNA Binding Matrix Solution® (Bio101 Fast DNA SPIN Kit for Soil; Qbiogene Carlsbad, CA) as described by the manufacturer, with modifications. Briefly, DNA samples were separated into 400 µl aliquots, then combined with 3 vol of DNA binding matrix in a 15-ml conical bottom
centrifuge tube (Fisher Scientific). The DNA samples were mixed by inversion for 2 min and allowed to settle for 3 min. After which, 0.7 ml of DNA binding matrix was loaded onto spin filter (supplied with the kit), then centrifuged at 14,000 x g (Eppendorf centrifuge 5415) for 1 min at room temperature. The spin columns were washed with 0.7 ml of (kit supplied) NaCl-Ethanol Wash Solution (SEWS, Bio101), using centrifugation as above. The matrix was allowed to dry for 5 min. The DNA was eluted with 60 µl of distilled water, using centrifugation as above. The DNA extracts were visualized as listed below.

**DNA gel electrophoresis.** The DNA eluted from hydroxylapatite and Sephadex G-75 columns was combined with 2X Gel Loading Dye (2 mM EDTA pH 8.0, 70% glycerol, 0.05% xylene cyanol, and 0.05% bromophenol blue; Bio Rad, Hercules CA), mixed thoroughly, then separated by electrophoresis on a 0.8% agarose gel. Agarose gel electrophoresis was performed using either a mini gel (76 mm x 50 mm) horizontal gel unit (H5, Gibco-BRL, Bethesda, MD) or using a large (139 mm x 110 mm) horizontal gel unit (H6, Gibco-BRL). When using a mini-gel, the DNA samples were run at 100 volts for 30 min (5.8 v/cm) in 1X TAE (40 mM Tris, 20 mM acetate, and 2 mM EDTA pH 8.0) gel running buffer. When using the large gel, the DNA samples were run at 100 volts for 4 h (3.7 v/cm) in 1X TAE gel running buffer. The gels were stained with 2.5 µg/ml ethidium bromide for 30 min, and photographed under UV transillumination using a Gel Doc 2000 (Bio Rad).

Ten microliters of amplification product from all PCR reactions was combined with 10 µl of 2X Gel loading Dye (Bio Rad), mixed thoroughly, then separated by electrophoresis on a 1.2% agarose gel which were run at either; 100 volts for 30 min (5.8 v/cm) in 1X TAE gel
running buffer in a mini-gel (H5, Gibco-BRL), or 100 volts for 4 h (3.7 v/cm) in 1X TAE in large horizontal gel unit (H6-Gibco-BRL).

**DNA extraction from *E. faecalis***. *E. faecalis* CG110 was grown to an A<sub>600</sub> of 0.4 and harvested by centrifugation 3,000 x g on a Beckman bench-top centrifuge (Beckman TJ-6 centrifuge; TA-10 rotor) for 5 min at room temperature. The cell pellet was resuspended in 0.7 ml of 10 mM Tris-HCl (pH 8.0), then added to a 2-ml bead-beating vial containing: 0.7 g of 0.1 mm sterile silica beads, 0.7 ml Tris buffered (pH 7.0)-phenol (Sigma-Aldrich), 0.7 ml Tris-HCl (pH 8.0). Cells were lysed by using a Mini-Beadbeater® (BioSpec Products Inc) for 120 sec at 5,000 rpm. Following centrifugation at 14,000 x g (Eppendorf centrifuge 5415) for 8 min at 4°C. The aqueous phase was transferred to a new tube and traces of phenol were removed by extraction with 1 vol of chloroform-isoamyl alcohol (24:1) (Fisher Scientific, Chicago, IL). Samples were vortexed on high speed for 30 sec, then centrifuged at 14,000 x g (Eppendorf centrifuge 5415) for 8 min at 4°C. The DNA was precipitated from the aqueous phase by adding an equal volume 95% ethanol and 1/10 volume of 3.0 M sodium acetate (pH 5.2), then centrifuged at 14,000 x g (Eppendorf centrifuge 5415) for 15 min at 4°C. The resulting DNA pellet was washed with 70% ethanol and resuspended in 0.5 ml distilled water containing RNAse (Sigma-Aldrich) at a final concentration of 10 µg/ml (final concentration), then incubated for 30 min at 37°C. The DNA was precipitated with ethanol (as above) and resuspended in 200 µl of distilled water. The DNA purity was estimated by using A<sub>260/280</sub> absorbance ratios on a Beckman DU7400 spectrophotometer (Fullerton, CA.). Purified DNA concentrations were estimated by spectrophotometric scans between 220 and 320 nm.
**PCR.** PCR primer pair ORF13 F (5' GGG TAC TTT TAG GGC TTA GT 3') and ORF13 R (5' GGC TGT CGC TGT AGG ATA GAG 3') were designed using OLIGO 6.7 software (Molecular Biology Insights, Inc. Cascade, CO) to amplify a 589-bp region from ORF13 of Tn916. The *E. faecalis* V583 genome has recently been sequenced (85), and has been deposited with GenBank as accession number AE016830. Specificity of this primer pair was determined hypothetically using the genome sequences from *E. faecalis* V583 and *B. subtilis* 168 on the OLIGO 6.7 program. Positive controls for these primers included *E. faecalis* CG110 and *B. subtilis* AS504 genomic extracts (108). PCR was performed in a total reaction volume of 25 µl using an Applied Biosystems thermal cycler (GeneAmp PCR system 9600, Fremont, CA). Buffer conditions were optimized with a FailSafe PCR PreMix Selection Kit (Epicentre Technologies, Madison, WI) using 12.5 µl of 2x PreMix K, 2.5 U of *Taq* DNA polymerase (Promega, Madison WI), 1.0 uM Forward primer (ORF13 F) and 1.0 uM Reverse primer (ORF13 R) under the following cycling conditions: 5 min at 94°C, 25 cycles of 30 sec at 94°C, 30 sec at 55°C, 45 sec at 72°C, plus an additional 7 min at 72°C. PCR products were separated by agarose gel electrophoresis and visualized as previously described.

**PCR sensitivity.** The *E. faecalis* V585 genome has been sequenced and contains 3.22 x 10^6 bp (85). In these experiments, genome equivalents were derived from the following assumptions (i) the average molecular weight of a nucleotide base pair is 660 daltons (ii) an *A*_{260} measurement of 1.0 is equivalent to 50 µg/ml of double stranded DNA. Genomic DNA was extracted from pure culture *E. faecalis* CG110 and the concentration was determined as described previously (see DNA extraction from *E. faecalis*). PCR signal from reactions containing measured quantities of CG110 DNA was used to identify the minimum detection
sensitivity. Accordingly, PCR reactions containing 200, 100, 50, 25, 13 and 6 pg of DNA template were run under standard conditions, and reactions products were analyzed using agarose gel electrophoresis.

**Inhibition of PCR by soil components.** Following extraction and purification from mock inoculated soil, the DNA was combined with purified *E. faecalis* DNA, which were added directly to PCR reactions to evaluate the potential interference of humic acids and background DNA on amplification efficiency. PCR was performed using template DNA from *E. faecalis* CG110 at concentrations of 200, 100, 50, 25 and 6 pg/g respectively. After adding the CG110 template, 3 µl of DNA extracted from the mock inoculated soil was added to the PCR tube. PCR products were visualized as described previously (see gel electrophoresis of PCR products).

**PCR on *E. faecalis* CG110 DNA seeded soil.** The efficiency of these extraction procedures was evaluated by seeding soil with known amounts (as described in similar studies (88)) of *E. faecalis* CG110 DNA. The DNA was added to 10 g native soil samples at concentrations of 300, 150, 75, 37, 6 pg DNA/g soil. Saline solution was added to soil as a negative control. The DNA was extracted and purified from the microcosms using the method described earlier (see soil DNA extraction). PCR was performed using 3.0 µl of undiluted soil DNA extracts. PCR products were visualized as described earlier (see gel electrophoresis of PCR products).

**PCR on *E. faecalis* CG110 cell seeded soil.** To determine lysis efficiency, PCR was performed on DNA template extracted from native soil seeded with known concentrations of *E. faecalis* strain CG110. Wherein, *E. faecalis* strain CG110 was grown to approximately 10^8 CFU/ml, serially diluted and inoculated into 10 g of sterile soil at concentrations
corresponding to $10^5$, $10^4$, $10^3$ and $10^2$ CFU/g soil. DNA was extracted and purified from the soil as described previously in this manuscript (see DNA extraction from soil). Three microliters of this DNA template was added to standardized PCR reactions. PCR products were visualized as described previously (see gel electrophoresis of PCR products).

**Effluent-soil microcosms.** Sterile soil samples were prepared by the methods of Haack et al. (39) with modifications. The soil (100 g), described previously (see soil characteristics), was added to a 250-ml centrifuge bottle (lying horizontally) and sterilized by autoclaving at 121°C for 4 h. Effluent-native soil microcosms were prepared as sterile microcosms were, without autoclaving. The samples were allowed to cool for 24 h. Moisture content was maintained at 7.5% by adding sterile distilled water on a weekly basis to microcosms (to replacing water loss due to evaporation). Microcosms were exposed to sunlight in a laboratory windowsill at room temperature, with bottle lids kept loose to maintain aeration. Effluent-soil microcosms were prepared by adding 16 ml of swine effluent to 100 g of sterile and non-sterile (native) soil. The samples were mixed gently with a sterile spatula. On a weekly basis, culturable bacterial counts from each microcosm were enumerated on plate count agar (Difco) with and without 40 µg/ml tetracycline from a 10-fold dilution series of a 1-g soil sample. As experimental controls, 1 g of un-amended native soil and 1 ml of effluent alone were serially diluted and plated (in duplicate) onto plate count agar (Difco) with and without 40 µg/ml tetracycline. An additional 10 g was removed for DNA extraction weekly using the methods described previously (see DNA extraction from soil). Following extraction, 3 µl of undiluted DNA extract was added to PCR reactions and amplification was performed as described previously in this manuscript (see PCR section).
PCR products were visualized as described previously (see gel electrophoresis of PCR products).
RESULTS

In this study, a series of four experiments was designed to evaluate the DNA extraction efficiency of these methods and identify the essential factors in obtaining PCR quality DNA from soil. In the first experiment, PCR was performed on pure culture *E. faecalis* DNA to determine the minimum sensitivity of detection. To measure PCR inhibition from soil compounds, the second experiment was performed by extracting DNA from mock-inoculated soil and added this to PCR reactions containing pure culture *E. faecalis* DNA. The third and fourth experiments were designed to evaluate the overall DNA extraction efficiency of these methods. Wherein, PCR signal was assayed from soil seeded with either (Experiment 3) *E. faecalis* CG110 cells or DNA from pure culture *E. faecalis* CG110 (Experiment 4).

**PCR sensitivity.** In the first set of experiments, PCR product formation from *E. faecalis* CG110 DNA was used to establish a minimum threshold of detection for the PCR reaction, and to serve as copy number standards of Tn916-like elements. Following DNA purification from pure culture CG110, PCR on the resulting template yielded products of the expected size range (589-bp) from reactions containing 200, 100, 50, 25, 12.5 and 6.0 pg of *E. faecalis* genomic DNA (Fig. 2). The minimum sensitivity of detection in this assay was $1.7 \times 10^3$ genome equivalents (6 pg) of *E. faecalis* CG110 DNA as indicated by a faint but detectable band in lane 7 (Fig. 2).

**DNA purification.** Humic compounds from soil have been shown to interfere with spectrophotometric analysis (75, 93, 113). As was the case using these columns, $A_{260}$ values from soil extracted DNA were low ($< 0.05$), and thus, the DNA quantity or purity could not be determined spectrophotometrically. The crude DNA was initially separated from soil-derived humic substances using hydroxylapatite columns. The extracts were washed and
FIG. 2. PCR sensitivity. The DNA template was extracted from *E. faecalis* CG110

Lane assignments: 100-bp ladder, (Lane 1). PCR with positive control containing 200, 100, 50, 25, 12.5, 5.0 pg (Lanes 2 to 7), negative control (Lane 8).
subject to elution in sodium phosphate buffers at 10, 140, 475 mM (pH 6.8). Following
elution with 475 mM sodium phosphate, the DNA content from 14 individual fractions was
assayed by agarose gel electrophoresis. The DNA eluted in a peak spanning fractions 8-11
(lanes 8-11, Fig. 3), corresponding to about 40% of the column volume. The absence of
DNA and the presence of RNA from pooled-ethanol precipitated, 140 mM sodium phosphate
eluates (data not shown), indicated that the columns were not overloaded. DNA extracts
from hydroxylapatite columns were desalted and further purified with gel filtration
chromatography using Sephadex G-75 columns. Double stranded DNA eluted from
Sephadex G-75 columns in a broad peak spanning fractions 7-13 (lanes 8-13, Fig. 4). In
previous experiments, PCR was performed with nucleic acids precipitated from individual
Sephadex G-75 column fractions, and resulted in no product formation (data not shown).
Indicating that PCR inhibitory compounds were not removed using hydroxylapatite and
Sephadex G-75 columns alone, and made apparent that further purification was needed.
Thus, fractions 7-13 from these columns were pooled and subject to further purification.

For the final stage of soil DNA purification, the Bio101 DNA Binding Matrix was used.
Pooled Sephadex G-75 column fractions were combined with the proprietary DNA binding
matrix and following elution, PCR was performed on the resulting DNA template. It was
only after this stage of purification that PCR signal was detected from Tn916-like elements in
soil. Thus, hydroxylapatite and Sephadex G-75 column chromatography in combination with
the Bio101 DNA Binding Matrix, resulted in the removal of PCR inhibitory compounds from
soil DNA.
FIG. 3. Agarose gel electrophoresis of DNA extracted from soil following hydroxylapatite column chromatography. The lane numbers correspond to the column fractions which sample was taken.
FIG. 4. Agarose gel electrophoresis of DNA extracted from soil Sephadex G-75 gel filtration column. The lane numbers correspond to the column fractions which sample was taken.
To determine whether inhibitory compounds from soil were present in DNA extracts, undiluted DNA, extracted from 10 g of mock-inoculated soil, was mixed with purified *E. faecalis* CG110 DNA; PCR reactions were conducted on these mixtures. The inhibitory properties of soil DNA extracts were evaluated by comparing the PCR sensitivity using pure CG110 DNA as template to analogous PCR reactions where mixtures of soil DNA extracts and CG110 template were used. In these experiments, similar sensitivities were observed.

PCR products of the expected size (589-bp) were obtained from reactions containing 200, 100, 50, 25, 12.5 and 6.0 pg of *E. faecalis* CG110 template only (lanes 2-7, Fig. 5). PCR products of the expected size (589-bp) were also obtained when *E. faecalis* CG110 template (diluted as above), was mixed with extracted soil DNA (lanes 8-13, Fig 5). A Tn916-like gene sequence was amplified by PCR in the presence of background DNA at every dilution. However, a reduction of PCR signal was observed when soil extract was included in the reaction mix (lanes 8-13, Fig. 5). This may be the result of soil contaminants (those carried through purification stages) inhibiting *Taq* polymerase or the result of non-specific DNA from soil masking target amplification. The sensitivity in both cases, however, was approximately $1.7 \times 10^3$ cell equivalents of *E. faecalis* CG110 (compare lane 8 and 13, Fig. 5), indicating that soil derived PCR inhibitors were adequately removed using these procedures.

**Sensitivity of *E. faecalis*-DNA seeded soil.** To evaluate the efficiency of DNA extraction, soil was inoculated with measured concentrations of *E. faecalis* CG110 genomic DNA. Following DNA extraction from these microcosms, PCR bands of the expected size (589-bp) were obtained from reactions containing DNA template extracted from soil inoculated with 300, 150, 75, 37 and 6.0 pg of *E. faecalis* CG110 genomic DNA (Lane 3-7,
FIG. 5. Detection of PCR amplicon in the presence of soil DNA extracts. Lanes 2-7 show amplicon from reactions containing only template whereas lanes 8-13 show amplicon from reactions containing both template and soil DNA. Lane assignments: 100-bp ladder (Lane 1). PCR with positive control 200 pg (Lane 2), 100 pg (Lane 3), 50 pg (Lane 4), 25 pg (Lane 5), 12.5 pg (Lane 6), 5 pg (Lane 7) 200 pg *E. faecalis* genomic DNA (positive control). PCR with 200 pg, 100 pg, 50 pg, 25 pg, 12.5 pg and 5 pg of *E. faecalis* genomic DNA (positive control) plus DNA extracted from negative control soil (Lane 8-13), PCR negative control (Lane 14).
Fig. 6). The minimum level of detection in this experiment was $1.7 \times 10^3$ genome equivalents of *E. faecalis* CG110 (Lane 7, Fig. 6).

To confirm primer specificity, native soil was inoculated with saline solution and PCR was performed with the resulting DNA template. As shown in Lane 6- Fig 7, no detectable PCR signal was obtained from these reactions, indicating that this primer pair was specific for Tn916-like elements.

**Sensitivity of *E. faecalis* cell seeded soil.** To determine the efficacy of cell lysis during the extraction procedure, soil was seeded with varying concentrations of *E. faecalis* CG110 cells. Following DNA extraction from soil, PCR products of the expected size were obtained from reactions containing DNA template extracted from 10 g of soil seeded with $10^6$, $10^5$, $10^4$, $10^3$ CFU/g *E. faecalis* CG110 cells. The minimum level of detection in this experiment was $8.0 \times 10^2$ CFU/g soil of *E. faecalis* CG110 (Lane 6, Fig. 8). The minimum detection thresholds obtained from pure CG110 DNA in combination with minimum detection sensitivities from both DNA and cell seeded soil, indicate that removal of inhibitory substances, DNA extraction, and cell lysis steps were efficient.

**Effluent-soil microcosms.** For a period of 6 weeks, a 1-g sample of effluent treated native soil, effluent treated sterile soil, effluent alone and non-sterile soil, were serially diluted and enumerated weekly on plate count agar with and without tetracycline (40 ug/ml). An additional 10 grams from these microcosms was removed weekly and subject to DNA extraction. PCR was performed with the resulting DNA template to estimate the abundance of Tn916-like elements. As shown in Fig. 9 and Table 2, culturable tetracycline resistant populations from effluent alone remained high ($10^5$ CFU/ml) from week 1 through week 6. In the absence of antibiotic selection, culturable cell counts from effluent alone were initially
FIG. 6. PCR from soil seeded with pure *E. faecalis* CG110 DNA. Amplification products from ORF13 region of Tn916. 100-bp ladder (Lane 1), 200 pg of *E. faecalis* DNA (Lane 2). Template used in these PCR reactions was DNA extracted from 10.0g of soil with the following amounts of *E. faecalis* CG110 DNA added: 3000 pg, (Lane 3); 1500 pg (Lane 4); 750 pg, (Lane 5); 375 pg, (Lane 6); 62.5 pg, (Lane 7); negative control, (Lane 8).
FIG. 7. PCR from negative control soil inoculated with 0.85% saline solution. Amplification products from ORF13 region of Tn916. 100-bp ladder (Lane 1), 62.5 pg of E. faecalis DNA (Lane 2). Template used in these PCR reactions was DNA extracted from 10.0 g of soil with the following amounts of E. faecalis CG110 DNA added: 3000 pg, (Lane 3); 1500 pg, (Lane 4); 750 pg, (Lane 5); 0 pg, (Lane 6); PCR negative control, (Lane 7).
FIG. 8. PCR from *E. faecalis* CG110 cell seeded soil. Amplification products from ORF13 region of Tn916. 100-bp ladder, (Lane 1). Template used in these PCR reactions was DNA extracted from 10.0 g of soil with *E. faecalis* CG110 cells added in the following concentrations: $10^6$ CFU/g soil, (Lane 2); $10^5$ CFU/g soil, (Lane 3); $10^4$ CFU/g soil, (Lane 4); $10^3$ CFU/g soil, (Lane 5). PCR negative control (Lane 6).
10^8 and 10^7 CFU/ml, and remained at this level throughout the sampling period. Indicating that both antibiotic resistant and susceptible populations remain culturable for at least 6 weeks in the aqueous swine lot effluent. Culturable tetracycline resistant populations from non-sterile soil were undetectable throughout the sampling period, while numbers on tetracycline-free plates ran at 10^6 and 10^7 CFU/ml throughout the sampling period. This was expected from a soil with minimal exposure to animal waste. Numbers of culturable tetracycline resistant bacteria from both effluent treated native and sterile soil microcosms (on plate count agar) declined sharply over the sampling period from approximately 4.0 x 10^6 and 5.9 x 10^5 CFU/ml to 10^3 CFU/ml after 28 days (Fig. 9 and Table 2). Plate counts from low dilutions (10^-1 and 10^-2) were hampered by soil debris and fungal mycelia, and resulted in a lower countable limit of ~10^3 CFU/ml, which was reached after 28 days post swine lot effluent application. In contrast, plate counts from effluent native microcosms on media without tetracycline declined slightly from their initial values of approximately 10^8 to 10^7 CFU/ml over the sampling period, while numbers of culturable bacteria from effluent treated sterile microcosms showed a similar slight decrease from approximately 10^8 to 10^7 CFU/ml after 35 days (Table 2 and Fig 10). Plate counts on media without tetracycline revealed that tetracycline susceptible bacteria in swine lot effluent remain culturable in sterile soil for at least 35 days post application. To detect the presence of Tn916-like gene sequence in effluent-soil microcosms, PCR was performed on DNA template extracted from both effluent treated native and sterile microcosms. As shown in Fig. 11, PCR signal intensities of Tn916-like elements from effluent treated native and sterile microcosms declined at different rates. For instance, during week 4, the PCR signal intensity from effluent treated sterile soil microcosms was greater than that of effluent treated native soil microcosms. Similarly,
FIG. 9. Tetracycline resistance microorganisms in soil microcosms. Plate counts from effluent added to sterile soil and non-sterile soil on plate count agar with tetracycline (40 µg/ml). Symbols: ■, culturable populations from effluent added to sterile soil; ◆, culturable populations from effluent added to nonsterile soil.
Table 2. Comparison of microcosm culturable cell numbers and PCR product from week 1 through week 6.

<table>
<thead>
<tr>
<th>Microcosm</th>
<th>Tetracycline</th>
<th>No Tetracycline</th>
<th>PCR product</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>week 1 week 6</td>
<td>week 1 week 6</td>
<td>week 1 week 6</td>
</tr>
<tr>
<td>Effluent</td>
<td>$10^6$</td>
<td>$10^8$</td>
<td>$10^7$</td>
</tr>
<tr>
<td>Non-sterile soil + Effluent</td>
<td>$10^6$ &lt;10$^3$</td>
<td>$10^8$</td>
<td>$10^7$ +</td>
</tr>
<tr>
<td>Sterile soil + Effluent</td>
<td>$10^5$ &lt;10$^3$</td>
<td>$10^8$</td>
<td>$10^7$ +</td>
</tr>
<tr>
<td>Non-sterile soil</td>
<td>&lt;10$^3$</td>
<td>$10^8$</td>
<td>$10^7$ --</td>
</tr>
</tbody>
</table>
FIG. 10. Total culturable microorganisms from microcosms. Plate counts from effluent added to sterile soil and non-sterile soil on plate count agar without tetracycline. Symbols:

■, culturable populations from effluent added to sterile soil; ◆, culturable populations from effluent added to nonsterile soil.
during week 5, the PCR signal from effluent treated sterile soil microcosms was greater than that of effluent-treated native soil microcosms. After week 6, PCR signal was detected from DNA extracted from effluent treated sterile soil microcosms (Table 2 and lane 13, Fig. 11), while this signal was absent in PCR reactions containing DNA extracted from analogous native soil microcosms (Lane 14, Fig. 11). Based on the detection threshold of Tn916-like elements established in control experiments, the copy number of Tn916-like elements in effluent-treated sterile soil microcosms after week 6 were, at minimum, \(1.7 \times 10^3\) genome equivalents, while copy numbers of Tn916-like elements in native soil microcosms ran at \(1.7 \times 10^3\) genome equivalents. The decline of PCR signal intensity from Tn916-like elements in effluent-treated native and sterile soil microcosms were similar to the reduction of culturable tetracycline resistance populations observed on plate counts.
FIG. 11. PCR amplification from effluent treated soil microcosms from week 1-6. 100 bp ladder, (Lane 1); PCR with DNA template extracted from: *E. faecalis* CG110, (Lane 2); week 1 effluent-sterile soil, (Lane 3); week 1 effluent-native soil, (Lane 4); week 2 effluent-sterile soil, (Lane 5); week 2 effluent-native soil, (Lane 6); week 3 effluent-sterile soil, (Lane 7); week 3 effluent-native soil, (Lane 8); week 4 effluent-sterile soil (Lane 9); week 4 effluent-native soil, (Lane 10); week 5 effluent-sterile soil, (Lane 11); week 5 effluent-native soil, (Lane 12); week 6 effluent-sterile soil, (Lane 13); week 6 effluent-native soil, (Lane 14).
DISCUSSION

**PCR from pure culture.** The size of the *E. faecalis* V583 genome has recently been determined (3.12 x 10^6 bp), which allowed for the conversion purified CG110 DNA concentrations (as determined by A_260 measurements) to genome equivalents. The *E. faecalis* CG110 genome contains one copy of Tn916 and the primer pair used in these experiments was specific for the ORF13 of this transposon. Thus, PCR product formation from pure *E. faecalis* CG110 DNA provided the bases for quantification of Tn916-like elements in both cell and DNA seeded soil and effluent soil microcosms. The detection threshold was determined by adding known quantities of pure DNA from *E. faecalis* CG110 to standardized PCR reactions. In the first experiment, a baseline detection sensitivity of 1.7 x 10^3 genome equivalents (or copies of Tn916-like elements) was established using PCR.

In determining the correct Epicentre® Premix to use for a standardized PCR reaction, the amount of *E. faecalis* DNA template was at saturating concentrations, which resulted in saturated PCR products in all Premixes assayed. Upon further analysis, the use of Premix K in these PCR reactions could have resulted in increased false priming, due the high end of magnesium chloride concentration found in Premix K.

**PCR inhibition.** To detect specific bacterial gene sequences from soil using PCR, separation of the compounds that typically co-extract with DNA must be achieved. In this protocol, the extracts were subject to a multi-step purification procedure that relied on the following: polyvinylpolypyrrolidone- to remove humic acids, phenol/chloroform- to extract proteins and nucleases, hydroxylapatite column chromatography- to separate DNA from humic acids, Sephadex G-75 gel filtration columns– to desalt and further purify nucleic acids
and reverse phase chromatography using the Bio101 DNA binding matrix- to remove humic acids from soil DNA extracts.

Prior to running HTP columns, DNA samples were heated to 42°C in a water bath to prevent salt precipitation. In addition, these columns were run on the laboratory bench at room temperature. Upon further analysis, allowing DNA to remain at room temperature during this stage of purification, in combination with the dissimilar temperatures between sodium phosphate buffers and hydroxylapatite columns, may have unwantingly exposed the DNA to bacterial nucleases released upon extraction. This may account for the extensive degradation of the DNA observed (<4000-bp).

Several reports show that spectrophotometric analysis of crude soil DNA is unreliable for quantitative analysis (75, 93, 113). Due to the presence of contaminating substances, the DNA yield after these purification procedures could not be determined using A260 values. Sephadex G-75 fractions 7-14 were pooled and subject to reverse phase chromatography using the Bio101 DNA binding Matrix. Based on PCR results from individual Sephadex fractions (data not shown), the combination of hydroxylapatite and Sephadex G-75 columns did not remove inhibitory substances from soil DNA. In gel-filtration chromatography, molecules are collected in order of decreasing molecular weight. Thus, pooling the latter fractions may have retained lower molecular weight fulvic acids in column eluates, and subsequently carried them through to PCR, resulting in a negative PCR reaction.

To further evaluate purification, DNA template from uninoculated native soil was assayed for purity using a PCR inhibition assay (74). In this experiment, the purified DNA extracts were added to PCR reactions containing measured amounts of pure *E. faecalis* DNA. The amplification of 6.0 pg (1.7 x 10³ genome equivalents) of *E. faecalis* DNA in the
presence of undiluted non-target DNA template (extracted from 10.0 g of soil), indicate that these purification procedures were efficient at removing contaminating substances from soil DNA. The DNA was suitable for PCR analysis only after silica based purification. The result of obtaining the minimal inhibition of positive control DNA template from soil DNA, indicate that this combination of purification procedures resulted in the effective removal of PCR inhibitors from soil.

**Lysis of* E. faecalis *in soil.** The detection of specific bacterial genes in soil requires cell lysis and the release of cellular DNA. The goal of this experiment was to (i) develop a method to achieve cell lysis of the Gram positive *E. faecalis* within soil and following purification and (ii) to be able to use this DNA to monitor for Tn916-like elements in soil microcosms.

In experiment 3, purification procedures and extraction efficiency were evaluated by inoculating $10^3$ CFU/g *E. faecalis* genome equivalents into the soil slurry immediately after sample homogenization. The rationale being; target DNA (released during homogenization) absorbing to soil components during the DNA extraction procedure, or target DNA potentially lost during purification, would result in a decrease in PCR signal, compared with PCR signal from DNA extracted from cell seeded soil (Experiment 4). Following extraction and purification, PCR was performed and a reduction of signal was not observed. Furthermore, if cell lysis was not complete, then PCR signal from cell seeded soil would be less than PCR signal from DNA seeded soil. This was not the case in these experiments. In experiment 3 (DNA-seeded soil) and experiment 4 (cell-seeded soil), comparable detection sensitivities were obtained; $1.7 \times 10^3$ CFU/g *E. faecalis* genome equivalents CG110 and 8.0 \times 10^2 *E. faecalis* CFU/g soil, respectively. Confirming that i) *E. faecalis* cells were lysed in
soil using the bead-beating method and ii) that these purification procedures removed PCR inhibitors from soil. The PCR signal from cells inoculated into soil at densities of $\sim10^6$ CFU/ml was greater than signal intensities from \textit{E. faecalis} CG110 DNA-inoculated soil corresponding to $\sim10^6$ genome equivalents. This could be the result of potential target DNA binding to clay or organic material in the soil during extraction, in essence, sequestering it from PCR analysis, or more likely, the result of bacterial nuclease degradation (that would have occurred during hydroxylapatite column purification at room temperature during the extraction procedure). The results from experiment 3 and 4 are in agreement with similar studies by Kuskie \textit{et al.} (59), who reported efficient cell lysis of \textit{Bacillus globuli} spores in soil, and Cresswell \textit{et al.} (21), who successfully lysed \textit{Streptomycetes} spores in soil using the bead-beating method. In a separate microcosm, a negative PCR with DNA template derived from mock-inoculated (or native soil) soil confirmed the specificity of this primer pair.

\textbf{Effluent soil microcosms.} Typically, 25-250 colonies can be counted on plates. Due to presence of fungal mycelia and soil particles on $10^{-1}$ and $10^{-2}$ plates, tetracycline resistant colonies on PCA plates could not be enumerated below $10^3$ CFU/ml. Plate counts of tetracycline susceptible populations from effluent-treated sterile soil microcosms suggest that these populations remain viable in sterile soil for a minimum of 6 weeks. In sterile soil, the survivability of introduced effluent microbial populations may be due to their ability to occupy the habitats previously occupied by native soil microbial populations.

Effluent-treated soil microcosms were prepared based on a typical application ratio of 64,000 lb/acre. Depending on the type of manure application system used (ie. injection, chisel, disk-type) wastes are applied to farm soil at a range of depths (see Table 3, appendix B) and in an un-uniform manor. In determining the correct application ratio to use, a uniform
application ratio of 64,000 lb/acre (injected 24 inches into the soil) was assumed. This would result in an application ratio 1:125 (effluent to soil). Under these assumptions, waste injected to a depth of 6 inches in a uniform manor, would result in an application ratio of 1:29. In these microcosms, the application ratio of 1:6 was used based on a soil density of 1.30 g/cm³ and more accurately reflects effluent concentrations near the point of injection.

This experiment was designed to monitor Tn916-like gene sequences in effluent treated soil microcosms by PCR, and to determine the fate of this transposon following the introduction of swine wastes to native soil and sterile soil microcosms. Thus, the microcosms were designed to evaluate the gene transfer potential of Tn916-like elements between manure microbial populations and indigenous soil microbial populations. The level of Tn916-like elements in effluent-treated sterile and non-sterile soils was assayed weekly using PCR product formation in combination with plate counts on tetracycline.

The presence of Tn916-like elements from effluent-treated sterile soil microcosms was detectable after 42 days using PCR. More PCR product was obtained from DNA extracted from effluent-treated sterile soil microcosms, compared to PCR product from DNA extracted from effluent-treated native soil microcosms, from week 4 through week 6. This data in combination with plate counts from effluent-treated soil microcosms suggest that levels of Tn916 mirrored that of tetracycline resistant populations.
REFERENCES


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Genetic analysis of a tetracycline resistance element from *Clostridium difficile* and its


Table 3. Effluent to soil microcosms ratios (64,000 lb/acre) according to depth.

<table>
<thead>
<tr>
<th>Injection depth (inches)</th>
<th>Effluent to soil ratio</th>
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<tbody>
<tr>
<td>24</td>
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<tr>
<td>18</td>
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<td>12</td>
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<td>6</td>
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</tr>
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<td>3</td>
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