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Tet 42, a Novel Tetracycline Resistance Determinant Isolated from Deep Terrestrial Subsurface Bacteria[▽]

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Tet 42, a novel tetracycline resistance determinant from deep subsurface bacteria, was characterized and found to have a 30% sequence similarity to TetA(Z). The protein is a putative efflux pump that shares characteristics with previously characterized pumps, including a divergently transcribed TetR repressor, a conserved GxxSDRxGRR motif, and transmembrane domains.

Tetracycline is a broad-spectrum antibiotic that binds to the 30S ribosomal subunit and inhibits translation by preventing the binding of tRNA. Tetracycline has been used clinically for more than 50 years, but the emergence of resistance has rendered it increasingly less useful. Tetracycline resistance was first characterized in the mid-1950s (3). Since then, more than 40 classes of resistance genes have been described, most of which confer resistance through active efflux (http://faculty.washington.edu/marilynr/tetweb4.pdf). Tetracycline resistance genes have been identified in bacteria from many environments (7, 14, 17), but little is known about resistance genes in terrestrial subsurface environments.

In the 1990s, sediments were extracted from 170 to 210 m below land surface at the U.S. Department of Energy Hanford Site (HS) in Washington State. Bacteria isolated from these sediments (6, 8) were later screened for resistance, and resistance to several antibiotics, including tetracycline, was reported (2). This study focused on the HS subsurface, because it has been isolated from surface influence for approximately 3 million years (23). The bacteria in these sediments have never been exposed to manmade antibiotics or to resistance genes that other bacteria have acquired through selective pressure from exposure to antibiotics. The aim of this study was to determine the genetic basis of tetracycline resistance in bacteria from this isolated environment.

The bacteria investigated in this study initially were found to be tetracycline resistant by plating on selective media containing 16 μg/ml tetracycline (2). MICs (Table 1) were then determined using a standard serial broth microdilution method (4).

Total DNA was extracted from strains G880, G887, G896, and G994 by using the 500G Genomic Tip kit (Qiagen, Valencia, CA). For strains G938, G983, and G985, cells were disrupted with the FastPrep-24 instrument (MP Biomedicals, Solon, OH) before extraction of DNA by a slight modification of the Genomic Tip protocol.

Chromosomal DNA was partially digested with various blunt-end restriction enzymes (New England Biolabs Inc., Ipswich, MA), and digests yielding fragments of 4 to 10 kbp were purified and concentrated by ethanol precipitation. Purified DNA digests were ligated into the pEZSeq vector (Lucigen Corporation, Middleton, WI) and transformed into electrocompetent cells (10G Elite; Lucigen) as described in the pEZSeq manual. The recovered cells were plated (100 μl/plate) onto yeast-tryptone agar plates with 16 μg/ml tetracycline. Resistant transformants were replated onto media containing tetracycline in order to determine the highest MIC for the resistance gene by using the standard agar dilution method (4). The MIC of the vector, without an insert, was determined by plating on media with 8 and 16 μg/ml tetracycline.

Clone plasmid DNA was screened using a double digest and run on a 1% agarose gel to identify clones containing an insert. DNA was sequenced as described in the supplementary methods to the article by Margulies et al. (12), with slight modifications as specified by 454 Life Sciences Corporation (Branford, CT).

Sequences were initially evaluated for homology to known tetracycline resistance determinants through a BLAST search. Sequence alignments and relatedness to homologous proteins were determined with the GCG SeqLab (version 11.1.3; Accelrys Software Inc., San Diego, CA) and PAUP (version 4.0 beta; D. Swofford, Sinauer Associates, Sunderland, MA) programs, respectively. Locations of conserved motifs were analyzed with the SMART website (9, 19).

DNA and protein sequence analysis. All seven strains possessed the same nucleotide base sequence for the resistance determinant described below. This sequence included two divergently transcribed open reading frames (ORFs) with low similarity to known proteins. The first and shorter ORF (612 nucleotides) was similar to the *tetA* repressor *tetR*. The second ORF (1,287 nucleotides) had a low similarity to previously characterized tetracycline efflux pumps. There is a 71-bp region between the two ORFs.

The larger ORF predicted a 429-amino-acid sequence most similar to TetA(Z) (21), with 30% homology (Fig. 1). The predicted amino acid sequence is <80% similar to any previously described tetracycline resistance determinant and was given the name TetA(42) according to current naming standards (11).

Ten transmembrane domains, located at amino acids 19 to 41, 51 to 73, 85 to 107, 112 to 131, 143 to 165, 169 to 191, 228

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TABLE 1. Bacterial strains examined in this study[a]

Strain	16S rRNA accession no.	Sediment sample	Sediment depth interval (m)	Lithofacies[a]	Nearest GenBank relative	Accession no. for nearest relative	Similarity score[b]	MIC (μg/ml)
G880	EU446126	YBO2-14	172.9–173.8	Lacustrine	*Bacillus thuringiensis* (T)	AF290545	1.000	16
G887	EU446133	YBO2-14	172.9–173.8	Lacustrine	*Microbacterium oxydans* 15E	DQ417333	1.000	32
G896	EU446137	YB02-26	183.1–183.9	Lacustrine	*Staphylococcus saprophyticus* subsp. *saprophyticus*	L37596	0.930	16
G938	EU446167	YB02-24	181.8–182.3	Lacustrine	*Paenibacillus jamilae* (T)	AJ271157	0.944	16
G983	EU446209	YB02-41	197.6–197.7	Fluvial gravel	*Pseudomonas pavonaceae* IAM1155	D84019	0.980	16
G985	EU446211	YB02-41	197.6–197.7	Fluvial gravel	*Pseudomonas pavonaceae* IAM1155	D84019	0.989	16
G994	EU446220	YB02-41	197.6–197.7	Fluvial gravel	*Pseudomonas pavonaceae* IAM1155	D84019	0.988	16
E. cloni Replicator Electrocompetent cells with the pEZSeq cloning vector								
Without Tet 42								4
With Tet 42								32

[a] As described by van Waasbergen et al. (23).
[b] Percent sequence identity over all pairwise comparable positions ($n = 8$) based on a partial 16S rRNA sequence (~730 bases).

to 250, 260 to 282, 291 to 313, and 378 to 400, were predicted for TetA(42). In contrast, previously reported efflux pumps contain 12 or 14 transmembrane domains (16). TetA(42) also contains the GxxSDRxGRR motif common to all tetracycline pumps (18). This region in TetA(42) (GrlSDRfGRR) is identical to that in TetA(Z) (Fig. 1).

The TetR(42) sequence comprises 204 amino acids and has a 41% amino acid similarity to the TetR transcriptional regulator from *Streptomyces ambofaciens* (1).

The intergenic region between *tetA* and *tetR* contains two TetR binding operators and promoters for both genes (16, 18). The putative operators (CGACAGTCTATCG and GACTAC ACTTTCG) are not perfect palindromic sequences and differ by 7 bases (Fig. 2). Similar variations have been seen in other determinants (22). The second operator, while not very similar to the first sequence, matches the second half of the first operator except for 1 base. The putative promoters are also shown in Fig. 2.

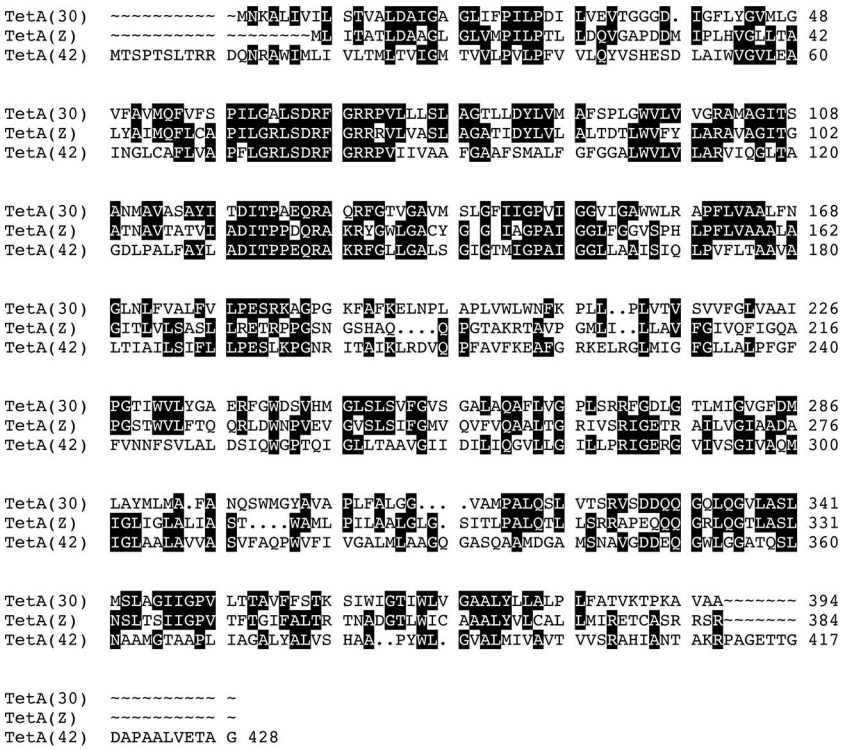

FIG. 1. Amino acid sequence comparison for TetA(42) and the two most closely related sequences, TetA(Z) and TetA(30). White letters on a black background indicate amino acids that are identical in two of the three sequences. GenBank accession numbers for TetA(Z) and TetA(30) are AAD25063 and AAD09860, respectively.

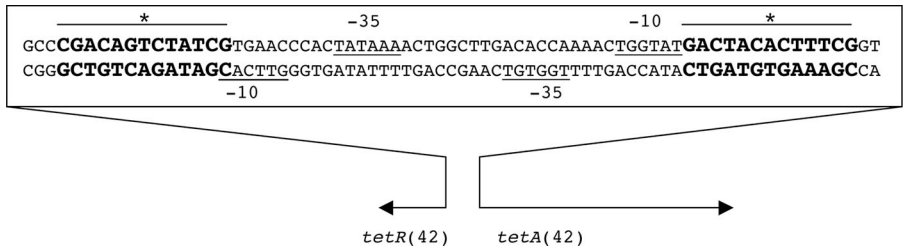

FIG. 2. DNA sequence of the 71-bp *tetA*(42)–*tetR*(42) intergenic region. Putative TetR(42) binding operators are in boldface and overlined. Each asterisk indicates the center of the inverted repeat for an operator. The −35 and −10 potential promoter sequences are indicated.

A phylogenetic analysis of the protein sequences of TetA(42) and representatives of each class of previously reported efflux pumps (Fig. 3) clearly distinguished TetA(42) from the other proteins by placement on a separate deep branch of the tree. The most closely related proteins were TetA(Z) and TetA(30).

The MICs for the subsurface strains ranged from 16 to 32 μg/ml (Table 1). Resistant transformants with the Tet 42 determinant showed an increase in the MIC from 4 to 32 μg/ml compared to the wild type (Table 1). The GenBank accession number for the DNA sequence of this class A determinant is EU523697.

Concluding remarks. The current study presents another tetracycline resistance determinant, Tet 42, that most likely confers resistance through active efflux. This determinant shares certain distinctive characteristics with previously reported tetracycline efflux pumps but has a low sequence similarity to the most closely related determinants. The bacteria investigated in this study were recovered from sediments that have been isolated from surface influence for approximately 3 million years (23), and as a result, they have never been exposed to commercial antibiotics or to bacterial genes that evolved in response to exposure to these antibiotics. It is not surprising, then, that the subsurface strains possess a novel resistance determinant.

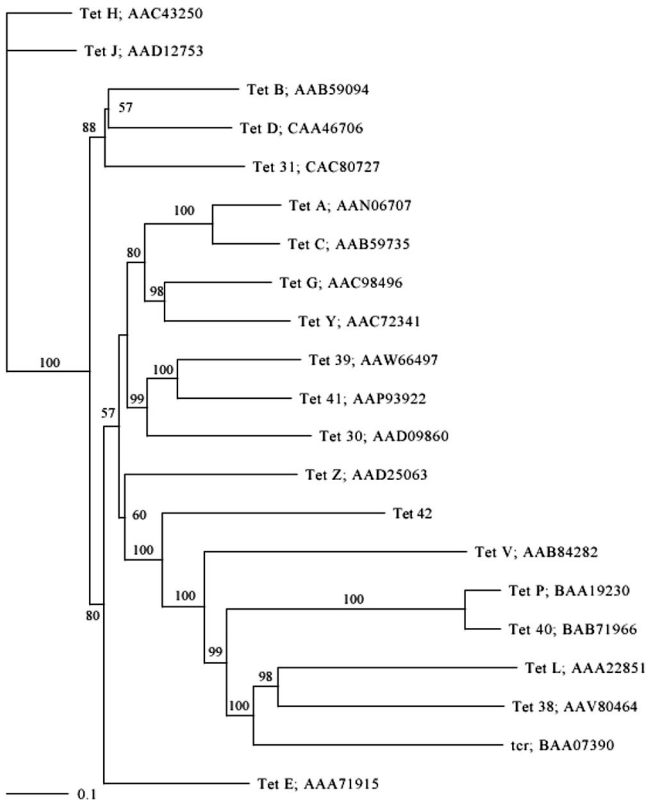

FIG. 3. Phylogenetic tree based on distance matrix analysis of amino acid sequences for Tet 42 and representative tetracycline resistance determinants that have been reported previously. GenBank accession numbers are given to the right of each resistance determinant. Scale bar, 10 substitutions per 100 amino acids.

The Tet 42 sequences were identical in all seven strains, which were isolated from several different sediment depths and included both gram-negative and gram-positive genera (Table 1). This finding raises interesting questions as to whether horizontal gene transfer (HGT) has occurred in the HS sediments. Smets et al. (20) demonstrated HGT in laboratory microcosms of subsurface sediment, and Martinez et al. (13) found evidence for HGT of lead resistance in subsurface soils. It is also interesting that these bacteria possess antibiotic resistance when they have been isolated from exposure to manmade antibiotics, unless antibiotics are being produced in the subsurface. Alternatively, they could possess an efflux pump to rid the cell of heavy metals or other toxins. The new resistance determinant presented here should help to further understanding of how TetA and TetR function, as well as providing useful information on antibiotic resistance in nature and the evolution of resistance genes. The discovery of a novel resistance gene in subsurface bacteria supports the suggestion that a pool of uncharacterized resistance mechanisms exists in environments that have not yet been examined in this regard (10, 15, 17).

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REFERENCES

1. **Barona-Gómez, F., S. Lautru, F. X. Francou, P. Leblond, J. L. Pernodet, and G. L. Challis.** 2006. Multiple biosynthetic and uptake systems mediate siderophore-dependent iron acquisition in *Streptomyces coelicolor* A3(2) and *Streptomyces ambofaciens* ATCC 23877. Microbiology **152:**3355–3366.
2. **Brown, M. G., and D. L. Balkwill.** Antibiotic resistance in bacteria isolated from the deep terrestrial subsurface. Microb. Ecol., in press.

3. **Chopra, I., and M. Roberts.** 2001. Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. Microbiol. Mol. Biol. Rev. **65:**232–260.
4. **CLSI.** 2003. Methods for dilution antimicrobial susceptibility test for bacteria that grow aerobically, CLSI document M7–A6, vol. 23, 2nd ed. Clinical and Laboratory Standards Institute, Wayne, PA.
5. Reference deleted.
6. **Crocker, F. H., J. K. Fredrickson, D. C. White, D. B. Ringelberg, and D. L. Balkwill.** 2000. Phylogenetic and physiological diversity of *Arthrobacter* strains isolated from unconsolidated subsurface sediments. Microbiology **146:**1295–1310.
7. **Furushita, M., T. Shiba, T. Maeda, M. Yahata, A. Kaneoka, Y. Takahashi, K. Torii, T. Hasegawa, and M. Ohta.** 2003. Similarity of tetracycline resistance genes isolated from fish farm bacteria to those from clinical isolates. Appl. Environ. Microbiol. **69:**5336–5342.
8. **Kieft, T. L., J. K. Fredrickson, J. P. McKinley, B. N. Bjornstad, S. A. Rawson, T. J. Phelps, F. J. Brockman, and S. M. Pfiffner.** 1995. Microbiological comparisons within and across contiguous lacustrine, paleosol, and fluvial subsurface sediments. Appl. Environ. Microbiol. **61:**749–757.
9. **Letunic, I., R. R. Copley, B. Pils, S. Pinkert, J. Schultz, and P. Bork.** 2006. SMART 5: domains in the context of genomes and networks. Nucleic Acids Res. **34:**D257–D260.
10. **Levy, S. B.** 1989. Evolution and spread of tetracycline resistance determinants. J. Antimicrob. Chemother. **24:**1–3.
11. **Levy, S. B., L. M. McMurry, T. M. Barbosa, V. Burdett, P. Courvalin, W. Hillen, M. C. Roberts, J. I. Rood, and D. E. Taylor.** 1999. Nomenclature for new tetracycline resistance determinants. Antimicrob. Agents Chemother. **43:**1523–1524.
12. **Margulies, M., M. Egholm, W. E. Altman, S. Attiya, J. S. Bader, L. A. Bemben, J. Berka, M. S. Braverman, Y. J. Chen, Z. Chen, S. B. Dewell, L. Du, J. M. Fierro, X. V. Gomes, B. C. Godwin, W. He, S. Helgesen, C. H. Ho, G. P. Irzyk, S. C. Jando, M. L. Alenquer, T. P. Jarvie, K. B. Jirage, J. B. Kim, J. R. Knight, J. R. Lanza, J. H. Leamon, S. M. Lefkowitz, M. Lei, J. Li, K. L. Lohman, H. Lu, V. B. Makhijani, K. E. McDade, M. P. McKenna, E. W. Myers, E. Nickerson, J. R. Nobile, R. Plant, B. P. Puc, M. T. Ronan, G. T. Roth, G. J. Sarkis, J. F. Simons, J. W. Simpson, M. Srinivasan, K. R. Tartaro, A. Tomasz, K. A. Vogt, G. A. Volkmer, S. H. Wang, Y. Wang, M. P. Weiner, P. Yu, R. F. Begley, and J. M. Rothberg.** 2005. Genome sequencing in microfabricated high-density picolitre reactors. Nature **437:**376–380.
13. **Martinez, R. J., Y. Wang, M. A. Raimondo, J. M. Coombs, T. Barkay, and P. A. Sobecky.** 2006. Horizontal gene transfer of PIB-type ATPases among bacteria isolated from radionuclide- and metal-contaminated subsurface soils. Appl. Environ. Microbiol. **72:**3111–3118.
14. **Patterson, A. J., R. Colangeli, P. Spigaglia, and K. P. Scott.** 2007. Distribution of specific tetracycline and erythromycin resistance genes in environmental samples assessed by macroarray detection. Environ. Microbiol. **9:**703–715.
15. **Riesenfeld, C. S., R. M. Goodman, and J. Handelsman.** 2004. Uncultured soil bacteria are a reservoir of new antibiotic resistance genes. Environ. Microbiol. **6:**981–989.
16. **Roberts, M. C.** 1996. Tetracycline resistance determinants: mechanisms of action, regulation of expression, genetic mobility, and distribution. FEMS Microbiol. Rev. **19:**1–24.
17. **Schmitt, H., K. Stoob, G. Hamscher, E. Smit, and W. Seinen.** 2006. Tetracyclines and tetracycline resistance in agricultural soils: microcosm and field studies. Microb. Ecol. **51:**267–276.
18. **Schnappinger, D., and W. Hillen.** 1996. Tetracyclines: antibiotic action, uptake, and resistance mechanisms. Arch. Microbiol. **165:**359–369.
19. **Schultz, J., F. Milpetz, P. Bork, and C. P. Ponting.** 1998. SMART, a simple modular architecture research tool: identification of signaling domains. Proc. Natl. Acad. Sci. USA **95:**5857–5864.
20. **Smets, B. F., J. B. Morrow, and C. Arango Pinedo.** 2003. Plasmid introduction in metal-stressed, subsurface-derived microcosms: plasmid fate and community response. Appl. Environ. Microbiol. **69:**4087–4097.
21. **Tauch, A., A. Puhler, J. Kalinowski, and G. Thierbach.** 2000. TetZ, a new tetracycline resistance determinant discovered in gram-positive bacteria, shows high homology to gram-negative regulated efflux systems. Plasmid **44:**285–291.
22. **Thompson, S. A., E. V. Maani, A. H. Lindell, C. J. King, and J. V. McArthur.** 2007. Novel tetracycline resistance determinant isolated from an environmental strain of *Serratia marcescens*. Appl. Environ. Microbiol. **73:**2199–2206.
23. **van Waasbergen, L. G., D. L. Balkwill, F. H. Crocker, B. N. Bjornstad, and R. V. Miller.** 2000. Genetic diversity among *Arthrobacter* species collected across a heterogeneous series of terrestrial deep-subsurface sediments as determined on the basis of 16S rRNA and *recA* gene sequences. Appl. Environ. Microbiol. **66:**3454–3463.